

"HUMANIZED" LARGE ANIMAL CANCER MODELS: ACCELERATING TIME AND EFFECTIVENESS OF CLINICAL TRIALS

EDITED BY: Kyle M. Schachtschneider, Gregers Jungersen,
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“HUMANIZED” LARGE ANIMAL CANCER MODELS: ACCELERATING TIME AND EFFECTIVENESS OF CLINICAL TRIALS

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“Translational Models”. Image: (top) Shutterstock.com/DedMityay,
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This eBook provides futuristic perspectives with respect to the emerging requirements of large animal cancer models to address unmet clinical needs. As the vast majority of drugs tested in small animal cancer models fail in human clinical trials, there is a need for large animal models to translate results obtained in small animal models to human clinical practice.

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Editorial: “Humanized” Large Animal Cancer Models: Accelerating Time and Effectiveness of Clinical Trials

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Editorial on the Research Topic

“Humanized” Large Animal Cancer Models: Accelerating Time and Effectiveness of Clinical Trials

In the United States alone, excluding contributions from governmental agencies and academic institutions, the private sector invests between US\$1.8–2.6 billion for each drug and between US\$75–94 million for each medical device development (1). From inception to regulatory approval, the development process requires ~13 years for drugs and 4.5 years for devices. Despite the time, cost, and effort, new therapies experience an 86–95% failure rate, primarily in the course of human clinical trials (1, 2). Cancer drugs seem to pose the greatest challenge with only 3.4–6.4% of those entering human clinical trials successfully advancing through to clinical use (2).

The potential failure rates, and the associated cost and time, can be mitigated if efficacy and safety of cancer drugs can be validated in translational preclinical animal models that mimic the complexities of the human disease, including comorbidities and confounding factors such as diet. Early discovery of unexpected hurdles allows for redesign and refinement prior to costly clinical testing. Of the three categories of animal models of human disease (3), *predictive models* (effects of a given treatment), *isomorphic models* (similar symptoms, different etiology), and *homologous models* (same symptoms, same etiology), the latter provide the greatest translational value when dealing with complex diseases such as cancers. Similarities to humans in anatomy, physiology, metabolism, immunology, and genetics is essential for recapitulating the interplay between various risk factors and molecular mechanisms of tumor development and progression. Here, an animal that is similar in size to humans adds critical value as it more closely models the size of tumors relative to organ and body size. This is not only important in modeling the spatial physiology of tumor microenvironments and the pathophysiological impact of developing tumors on the whole body, but also for accurately evaluating the efficacy and safety of promising novel therapies. Furthermore, human-sized models allow for the use of human clinical imaging modalities and medical devices during the pre-clinical phase of therapy development, enabling efficacy assessments to be made as they would be in the clinical setting.

As the vast majority of drugs tested in small animal cancer models fail in human clinical trials, there is a need for large animal models to accurately translate results obtained in small animal models to human clinical use, and also address unmet clinical needs. In addition, the majority of preclinical immunotherapy studies conducted in rodents have translated poorly to the clinic due to substantial differences between murine and human immunology. As the porcine and canine

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immune systems display substantial homology to that of humans, these large animals represent excellent platforms for preclinical investigation of cancer immunotherapies (4). Porcine (5) and canine (6) cancer models are thus rapidly gaining acceptance and popularity for use in cancer research, and are being recognized as valuable tools for testing of drugs and devices in co-clinical trials. The continued development of genomic and phenomic tools and databases also provides the ability through genome editing to create "humanized" experimental large animal models that can support interventional targeted cancer drug and device development. These large animal models also allow for the inclusion of relevant comorbidities such as alcohol-induced cirrhosis, non-alcoholic steatohepatitis (NASH), diabetes, obesity, and cardiovascular disease.

This *Frontiers in Oncology* special issue presents three original research articles, six reviews, and a hypothesis article spanning porcine, canine, and ovine cancer models. In the **Original Research** section, Gray et al. describe a novel naturally-occurring ovine pulmonary adenocarcinoma model to validate the ability of miniaturized implantable sensors to monitor tumor microenvironment by integrating techniques used in the treatment of human lung cancer patients. Schlein et al. use a canine model of naturally-occurring brain cancers, along with human tumor samples and cell lines, to validate procaspase-3 as a druggable target for specific brain tumors, particularly high grade astrocytomas. Boettcher et al. establish that xenotransplantation of human ovarian cancer into severe combined immune deficient (SCID) pigs phenotypically resembled human ovarian carcinomas and substantiate further development of orthotopic pig models.

In the **Mini Review** and **Review** sections, Gray et al. describe the advantages of using naturally occurring ovine pulmonary adenocarcinoma models, including their value in evaluating chemotherapeutic agents and monitoring the

tumor microenvironment. Faraji and Gaba review medical imaging modalities, current radiologic diagnostic criteria and response assessment schemes for evaluating therapeutic response and disease progression, and explore translation of radiologic imaging protocols and standards to large animal models of malignant disease. Bailey and Carlson describe a pancreatic tumor model utilizing Cre-inducible transgenic Oncopigs with KRAS and p53-null mutations to overcome the limited translational accuracy and utility of murine models. Duran-Struuck et al. highlight the advantages of swine models for the study of hematological malignancies and describe their experience with a transplantable tumor model that utilizes spontaneously arising tumors in MGH swine. Xu et al. discuss the utility of developing genetically defined porcine cancer models as clinically relevant, personalized cancer models for use in co-clinical trials, ultimately improving treatment stratification and translation of therapeutic approaches to clinical practice. Fan and Selting present the value of dogs with spontaneous tumors as a model to advance harnessing of abscopal effects for clinical use. That is, how radiotherapy could be used to trigger systemic anticancer immune activation, allowing for regression of cancerous lesions distant from the primary site of radiation delivery.

In the **Hypothesis and Theory** section, Boettcher et al. present the hypothesis that SCID pig models are well-suited for improved engraftment and differentiation of human immune cells, and how such humanized pig models can be used to study interactions between human tumors and human immune cells, and to develop patient-specific therapies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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A Novel Translational Ovine Pulmonary Adenocarcinoma Model for Human Lung Cancer

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In vitro cell line and *in vivo* murine models have historically dominated pre-clinical cancer research. These models can be expensive and time consuming and lead to only a small percentage of anti-cancer drugs gaining a license for human use. Large animal models that reflect human disease have high translational value; these can be used to overcome current pre-clinical research limitations through the integration of drug development techniques with surgical procedures and anesthetic protocols, along with emerging fields such as implantable medical devices. Ovine pulmonary adenocarcinoma (OPA) is a naturally-occurring lung cancer that is caused by the jaagsiekte sheep retrovirus. The disease has similar histological classification and oncogenic pathway activation to that of human lung adenocarcinomas making it a valuable model for studying human lung cancer. Developing OPA models to include techniques used in the treatment of human lung cancer would enhance its translational potential, making it an excellent research tool in assessing cancer therapeutics. In this study we developed a novel OPA model to validate the ability of miniaturized implantable O₂ and pH sensors to monitor the tumor microenvironment. Naturally-occurring pre-clinical OPA cases were obtained through an on-farm ultrasound screening programme. Sensors were implanted into OPA tumors of anesthetized sheep using a CT-guided trans-thoracic percutaneous implantation procedure. This study reports the findings from 9 sheep that received sensor implantations. Time taken from initial CT scans to the placement of a single sensor into an OPA tumor was 45 ± 5 min, with all implantations resulting in the successful delivery of sensors into tumors. Immediate post-implantation mild pneumothoraces occurred in 4 sheep, which was successfully managed in all cases. This is, to the best of our knowledge, the first description of the use of naturally-occurring OPA cases as a pre-clinical surgical model. Through the integration of techniques used in the treatment of human lung cancer patients, including ultrasound, general anesthesia, CT and surgery

into the OPA model, we have demonstrated its translational potential. Although our research was tailored specifically for the implantation of sensors into lung tumors, we believe the model could also be developed for other pre-clinical applications.

Keywords: human lung cancer, ovine pulmonary adenocarcinoma, novel translational lung cancer model, pre-clinical research, computed tomography-guided sensor implantation

INTRODUCTION

The process of developing and validating new anti-cancer agents typically follows a step-wise process from *in vitro* and *in vivo* testing through to phase I, II, and III clinical trials. It has been estimated that several hundred million dollars and up to 10 years of research is needed to take a drug from its initial concept to the completion of phase III trials (1). Pharmaceutical companies may be discouraged from developing new cancer drugs, not only due to the resources required, but also because attrition rates for new cancer therapeutics are very high. Only 5% of agents that show pre-clinical promise gain a license to be used in patients after phase III trials (2). While the use of *in vitro* techniques and *in vivo* murine models are well-established in pre-clinical cancer research, fewer large animal translational models have been described. These models show promise in overcoming current limitations in pre-clinical research by permitting the integration of drug development techniques with surgical procedures and anesthetic protocols, along with novel cancer therapeutic strategies such as implantable medical devices.

The use of implantable medical devices for cancer diagnosis, treatment, and monitoring is becoming attainable due to advances in electronics and microfabrication techniques. *In vivo* murine studies have already shown that implantable devices can be used to detect cancer secreted biomarkers (3) or to release chemotherapy drugs directly within tumors (4). Numerous other studies have also investigated the biocompatibility and functionality of implantable devices using *in vivo* (predominantly rodent) models for a range of other disease conditions, providing evidence of their increasing potential for clinical uses (5).

Lung cancer remains the most commonly diagnosed cancer in the world, with ~1.8 million new cases and 1.6 million cancer-related deaths recorded each year (6). Information on the molecular basis and pathogenesis of human lung cancer continues to grow through the use of numerous *in vitro* cell line and *in vivo* murine models (7–12). However, pre-clinical research using murine models has failed to improve overall survival rates, which remains low (~15%).

Comparative oncology is the use of naturally-occurring cancers that arise in veterinary species for the study of cancer biology and therapy (13); this approach is increasingly being used to reconcile the gap between *in vitro* experiments, *in vivo* small animal research and human clinical trials. Naturally-occurring tumors within veterinary species that have incidence rates or pathological similarities comparable to human cancers have considerable potential as translational models of human disease (14). Ovine pulmonary adenocarcinoma (OPA) is a naturally-occurring neoplastic lung disease caused by the jaagsiekte sheep retrovirus (JSRV) (15–18). The disease is regarded as a valuable translational pre-clinical research model for studying human

lung cancer, overcoming many of the limitations associated with current murine models (19).

The Implantable Microsystems for Personalized Anti-Cancer Therapy (IMPACT) programme (University of Edinburgh) is developing miniaturized implantable O₂ and pH sensors designed to monitor the tissue microenvironment within a solid tumor. The identification of hypoxic tumor regions should improve the ability to target these radiation and chemo-resistant areas (20). Each sensor is fabricated on a silicon chip and bonded to a 1.7 × 200 mm long flexible printed circuit board lead. The sensors are sealed in biocompatible epoxy resin, resulting in an overall sensor size of ~2.8 × 5.1 × 1.4 mm (width × length × height). The sensors are sterilized using ethylene oxide. We have capitalized upon a naturally-occurring OPA model in order to validate these sensors within a solid tumor. By integrating techniques used in the treatment of human lung cancer patients (ultrasound, general anesthesia, CT, and surgery) into the OPA model, we have shown its translational potential. Whilst our model was specifically developed for the implantation of sensors into solid tumors, we believe it has considerable potential for other pre-clinical studies.

MATERIALS AND METHODS

Studies were undertaken under a UK Home Office Project License in accordance with the Animals (Scientific Procedures) Act 1986 and with approval from the University of Edinburgh Animal Welfare and Ethical Review Boards. The recommended guidelines for welfare and use of animals in research were followed. Nine adult female sheep (Highlander, *n* = 1; Scottish blackface, *n* = 7; Scotch Mule, *n* = 1), weighing 39–65 kg and diagnosed with naturally-occurring pre-clinical OPA, were obtained through an on-farm ultrasound eradication programme (21, 22). Sheep were bedded on straw, with *ad libitum* access to food and water in groups of at least 2 animals and were allowed a period of adaptation of at least 24 h before undergoing anesthesia.

General Anesthesia

Anesthesia was managed by specialist veterinary anesthetists or by veterinary surgeons enrolled in a specialist training programme under supervision. All sheep underwent preanaesthetic assessment, which involved distant observation of demeanor, breathing rate and pattern, and was followed by physical examination. Only animals that were judged fit for anesthesia were subsequently studied. Food was withheld for 12 h before anesthesia, but access to water was permitted until preanaesthetic medication was administered. Anesthesia and analgesia techniques are provided in **Table 1**. Intravenous preanaesthetic medication was administered to reduce animal stress, facilitate the induction of anesthesia and to decrease

TABLE 1 | Techniques used to provide anesthesia and analgesia in sheep with ovine pulmonary adenocarcinoma in pre-clinical research.

Phase	Drug	Manufacturer	Dose (mg/kg)	Route
Sedation	Medetomidine	"Sedator"; Dechra Veterinary Products, Shrewsbury, UK	0.003–0.01	i.v.
	<i>in combination with</i>			
	Midazolam	"Hypnovel"; Roche, Welwyn Garden City, UK	0.25–0.5	i.v.
Induction	Propofol	"Propofol"; Fresenius Kabi, Cheshire, UK	To effect* (e.g., 3–10)	i.v.
Maintenance	Isoflurane	"IsoFlo"; Abbot Animal Health, Maidenhead, UK		inhaled
Analgesia	Flunixin	"Flunixin Injection"; Norbrook, Newry, UK	2.2	i.v.
	Morphine	"Morphine Sulfate"; Martindale Pharmaceuticals, Essex, UK	0.1–0.3	i.v./i.m.

*Until conditions for endotracheal intubation are present. (i.m., intramuscular; i.v., intravenous).

induction agent dose requirements. General anesthesia was induced within 10 min of preanaesthetic medication to minimize sedation-induced respiratory depression. Before this, and when necessary, the head was elevated to prevent respiratory secretions and rumen contents entering the upper airway. After induction of anesthesia, the trachea was intubated with a cuffed endotracheal tube and the cuff inflated. Anesthesia was maintained using isoflurane (Abbot Animal Health, Maidenhead, UK) vaporized in an O₂/air mixture, administered using a Bain or circle breathing system connected to the endotracheal tube. End-tidal concentrations of 1.5–2.0% isoflurane were used to ensure unresponsiveness to subsequent procedures. Oropharyngeal and tracheobronchial suction was performed to remove respiratory secretions when required. After tracheal intubation, the lungs were ventilated mechanically to achieve tidal volumes of 8–10 ml/kg. Respiratory rate was adjusted to maintain normocapnia (PaCO₂ range 4.7–6 kPa). Body temperature was monitored using rectal and esophageal thermistors and maintained between 38.5°C and 39.5°C. A central (jugular) venous 14G cannula was used for administering drugs and crystalloid fluids. Compound sodium lactate (Aqupharm No 11, Animalcare, York, UK) was infused at 5 ml/kg/h in order to sustain cardiac preload and replace lost fluids and electrolytes. Mean arterial blood pressure was maintained between 70 and 80 mmHg and monitored using an arterial cannula placed in the central auricular artery. Blood samples obtained from the arterial cannula was used for intermittent blood-gas, biochemical and hematological analysis (Epoc portable blood gas electrolyte and critical care analyser; Woodley Equipment Company Ltd, Lancashire, UK). A multiparameter patient monitoring device (Datex-Ohmeda S/5, SOMA Technology, Madison, USA) was used to continuously monitor pulse rate and blood pressure along with pulse oximetry, capnography, temperature, spirometry, electrocardiography and inspired and expired gases (O₂, CO₂, and inhalant anesthetic agent) (**Figure 1**). Analgesic agents were administered pre-emptively either at the time of sedation or immediately post-induction. All animals were euthanized with intravenous sodium pentobarbitone (Pentoject; Animalcare, York, UK).

Computed Tomography Imaging

A single-section SOMATOM Definition AS 64 slice helical CT machine (Siemens Healthcare Ltd, Camberley, UK) was used for all advanced imaging procedures. The imaging parameters of the

scanner were 120 kVp, 35 mA, 3–5 mm collimation with 1 mm section thickness. The window width and level were ~2,000 and –500 HU, respectively, allowing simultaneous visualization of the needle tip, blood vessels, OPA lesions, pneumothorax, bone, muscle, and fat. All scans were performed to include the entire thoracic cavity from the thoracic inlet to the last rib.

Development of a Trans-Thoracic Percutaneous Technique for Sensor Implantation Into OPA Tumors

The model was initially developed using cadavers of OPA-affected sheep; simulated surgeries were performed on 8 sheep cadavers with multiple sensor implantations in each carcass. These surgeries allowed the development of the implantation procedure and investigation of the potential accessible regions of the thoracic cavity and lung lobes into which sensors could be safely implanted. To refine the surgical procedure further, 3 sheep diagnosed with pre-clinical OPA by ultrasound screening underwent anesthesia and sensor implantation as developed from the cadaveric studies. Refinements to the procedure included the use of radiopaque grid lines for improved accuracy of lesion localization and performing serial CT scans to aid needle positioning and sensor implantation. These staged series of experiments allowed the development of our OPA model; each development stage increased the complexity of the model, resulting in the refined protocol used in experimental cases.

All experiments were conducted on anesthetized animals. After induction of anesthesia, sheep were placed in lateral recumbency with the diseased lung uppermost. The thorax was clipped between the caudal border of the last rib and the caudal border of the scapula. The dorsal margin extended from the dorsal spinous process of the thoracic vertebrae ventrally to the sternum. An initial CT scan was taken to assess intra-thoracic pathology and identify OPA lesions for implantation. Lesions were selected so the needle path would avoid bullae, fissures, visible blood vessels, and large bronchioles. Peripheral lung lobe lesions of at least 4 cm diameter were preferred to limit the volume of normal aerated lung that the needle would pass through and to improve sensor implantation into OPA tissue (**Figure 2**). To aid OPA lesion localization and determine the site for percutaneous sensor placement, initial CT scans were performed with a self-adhesive sheet of non-metallic, radiopaque grid lines (GuideLines, Oncology Imaging Systems, UK) placed

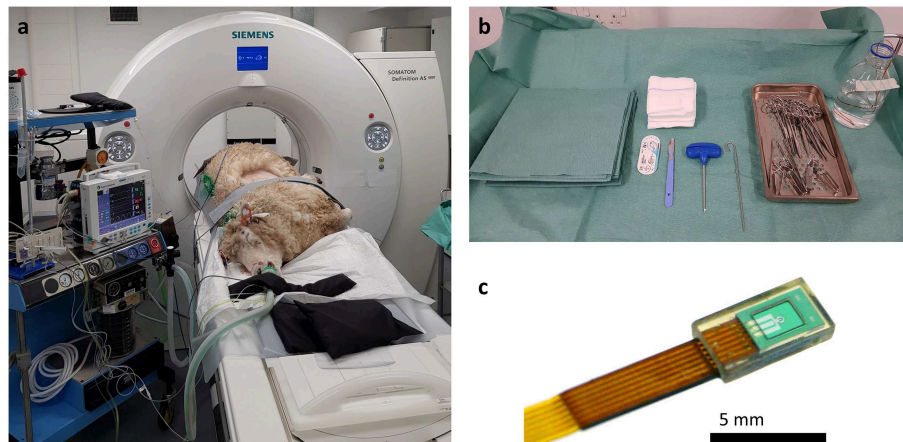


FIGURE 1 | Photographs of imaging and surgical facilities. **(a)** The sheep is anesthetized and positioned for CT scanning in lateral recumbency, with the OPA affected lungs uppermost. The anesthetic monitoring equipment can be seen to the left-hand side of the image. **(b)** For sensor implantation a general surgical kit is required along with the Jamshidi insertion needle. An IMPACT sensor can be seen in a bottle of sterile saline at the top right-hand side of the image. **(c)** Macroscopic image of the IMPACT O₂ sensor encapsulated in a biocompatible epoxy resin and bonded to the lead wire.

on the thoracic wall skin surface. OPA lesions were localized dorso-ventrally based on the grid lines and cranio-caudally based on intercostal spaces. The distance between the skin and pleura was measured at the anticipated penetration site. A mark was drawn on the skin surface to identify the position of thoracic wall penetration for sensor implantation. The grid lines were removed, and the skin was aseptically prepared for surgery using chlorohexidine solution, after which the area was four quarter draped for surgery (**Figure 3**).

All sensors were inserted using a trans-thoracic percutaneous technique under CT guidance. Based on the initial pre-operative CT scan a 1 cm vertical skin incision was made ~1–2 intercostal spaces caudal to the desired entry point into the thoracic cavity. An 8G × 15 cm Jamshidi biopsy needle (Carefusion, France), with its stylet in place, was advanced cranially through subcutaneous tissues, then redirected perpendicular to the thoracic wall in the center of the chosen intercostal space. The needle was advanced through the chest wall (based on the pre-measured distance from the initial CT scan), with the penetration of the parietal pleura appreciated as the feeling of a “pop.” The needle, at this point, was within the thoracic cavity through the parietal pleura, but not penetrating lung/OPA tissue. A second CT scan at this stage confirmed the position of the needle. If necessary, the needle could be repositioned with minimal risk of lung damage as the needle had not penetrated the visceral pleura. Once in the correct position the needle was slowly advanced through the visceral pleura into OPA tissue; repeat CT scans were taken following each needle advancement and measurements were made determining the distance from needle tip to the point of desired sensor implantation. Following placement of the needle tip centrally within OPA tissue, the stylet was removed from the Jamshidi needle and the sensor and lead wire were introduced down the bore of the needle. The obturator was then placed down the bore of the needle, advancing the sensor past the tip of the needle into OPA tissue. Once in place, the

obturator and implantation needle were withdrawn, leaving the sensor and lead wire *in situ*. A purse string suture of 3 metric braided silk (Mersilk, Ethicon UK), placed around the incision which continued as a Chinese finger trap suture around the lead wire, secured the sensor in place (**Figures 4, 5**). Final CT scans were performed to evaluate sensor positioning and assess any immediate post-operative complications such as pneumothorax or hemorrhage. The decision to drain any pneumothorax that developed (though percutaneous thoracocentesis) was made based on its severity. Post-mortem examination was performed following the completion of the experiments to assess the extent of lung pathology, identify the implant site and to obtain biopsy specimens for histopathology.

Histopathology

OPA tissue was fixed for at least 24 h (depending on tissue thickness) in 4% formaldehyde (Genta Medical, UK) before undergoing processing using the Thermo Scientific Excelsior AS Tissue Processor (Thermo Scientific, UK) and embedding in paraffin. Tissue was sectioned using the Leica RM2235 rotary microtome (Leica Microsystems Ltd, UK); microtome sections of 4 μm were placed on SuperFrost Plus glass slides (Thermo Scientific, UK) and allowed to dry for a minimum of 4 h at 53°C.

For haematoxylin and eosin staining, sections were deparaffinised by 3 changes in 100% xylene for 5 min, then rehydrated by placing into alcohol; 2 changes in 100% ethanol, followed by 80% then 50% for 2 min each time. The slides were washed in running water for 2 min, before placing in haematoxylin (Shandon Harris Haematoxylin, Thermo Scientific, UK) for a maximum of 10 min. Slides were washed in running water for 2 min and then placed into Scott's tap water substitute for a maximum of 10 min until the tissue sections turned blue. Sections were counterstained by placing them into Eosin Y (Shandon Eosin Y Cytoplasmic Counterstain, Thermo Scientific, UK) for 5 min. The slides were dehydrated by placing



FIGURE 2 | Initial CT images are used to assess intra-thoracic pathology. **(a)** Axial, **(b)** Coronal, and **(c)** Sagittal planes. A large area of increased radiopacity, consistent with an OPA lesion, can be seen affecting the ventral regions of the left cranial and caudal lung lobes (outlined in black). Air bronchograms are visible within this region (white arrows). An area of patchy and hazy increased opacity (ground glass appearance) within the dorsal regions of the lung, with preservation of bronchial and vascular patterns, can also be identified (outlined in white); this increased opacity may be due to the presence of diffuse areas of neoplastic foci or pneumonia. The radiopaque circles on the skin surface seen on the axial plane are the grid lines used for OPA lesion localization.

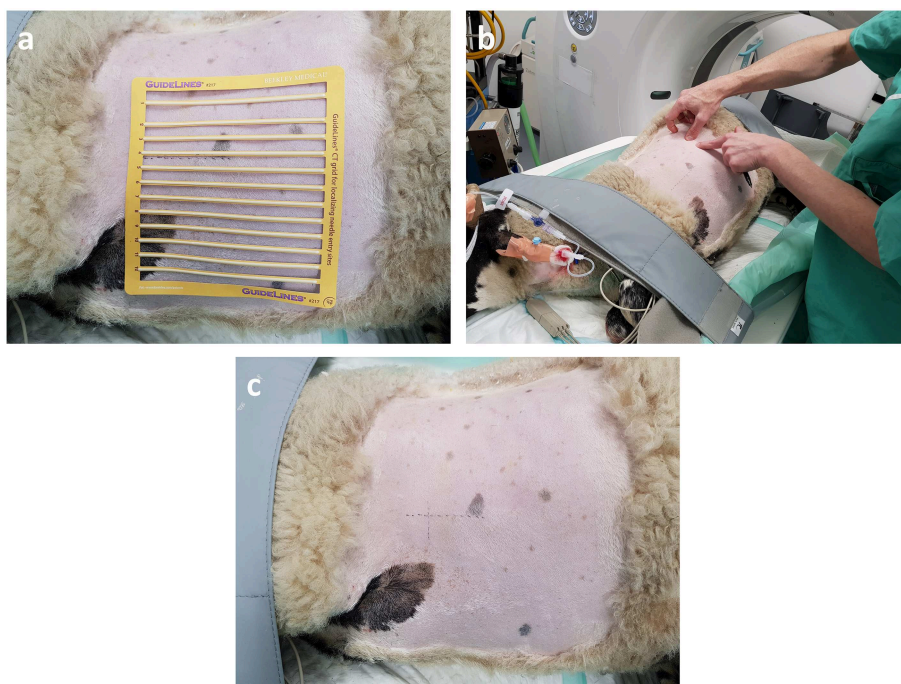


FIGURE 3 | OPA lesion localization. The hemithorax has been clipped for surgery and the radiopaque grid lines are placed on the skin surface. **(a)** The grid lines are placed on the skin surface prior to the initial CT scan. **(b,c)** The skin is marked both dorso-ventrally and cranio-caudally at the desired implantation point based on the initial CT images.

them into alcohol; 50% ethanol for 30 s, 80% ethanol for 30 s, then 2 changes in 100% ethanol for 2 min. The slides were placed in xylene for 10 min before being mounted with coverslips using DXP mountant (Sigma-Aldrich, UK).

Assessment of Radiation Exposure During CT-Guided Sensor Implantations

To assess the amount of radiation that sheep were exposed to during CT-guided sensor implantations, the total number of CT imaging events (topograms or full thoracic scans) were recorded

and individual imaging event and total dose length products (DLP) were calculated for each sheep. Individual event DLP is calculated from the CT dose index volume ($CTDI_{vol}$), which is in turn based on the radiation received inside a phantom from a single rotation of the scanner, this value is then multiplied by the scan length. Total DLP for each sheep was calculated from the sum of all individual DLP's. DLP is proportional to the effective dose received by a patient and is used, in combination with CTDI, to compare scanning protocols and establish diagnostic reference levels (23).

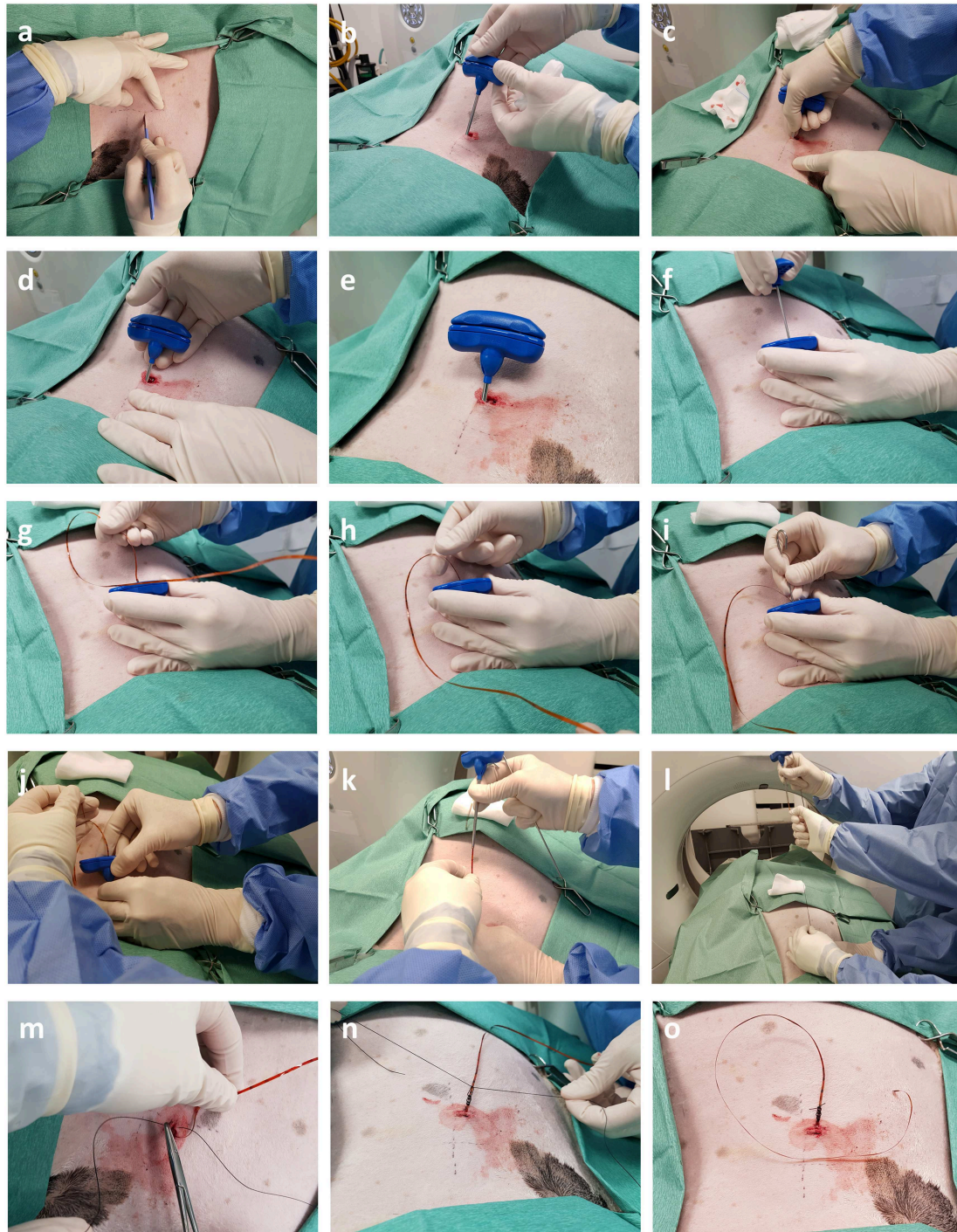


FIGURE 4 | Intra-operative photographs depicting trans-thoracic percutaneous sensor placement. **(a,b)** A skin incision is made through which the Jamshidi needle is introduced. **(c-e)** Following successive CT scans the needle is progressively advanced into OPA tissue. **(f-h)** Once the needle is in position the stylet is removed and the sensor introduced down the bore of the needle. **(i)** The obturator is used to push the sensor past the tip of the needle into OPA tissue. **(j-l)** The Jamshidi needle is removed, leaving the sensor and lead wire in place. **(m-o)** The skin is closed, and lead wire secured in place with a purse string and Chinese finger trap suture.

Statistical Analysis

Data for blood-gas, biochemical and hematological analysis was analyzed with parametric tests. One-way ANOVA with Holm-Šidák multiple comparisons test were used to test for differences

over time; p -values <0.05 were deemed statistically significant. Data are shown as mean $\pm$ SEM, with all statistical analysis and graphs generated using Prism 7 (GraphPad Software, San Diego, CA, USA).

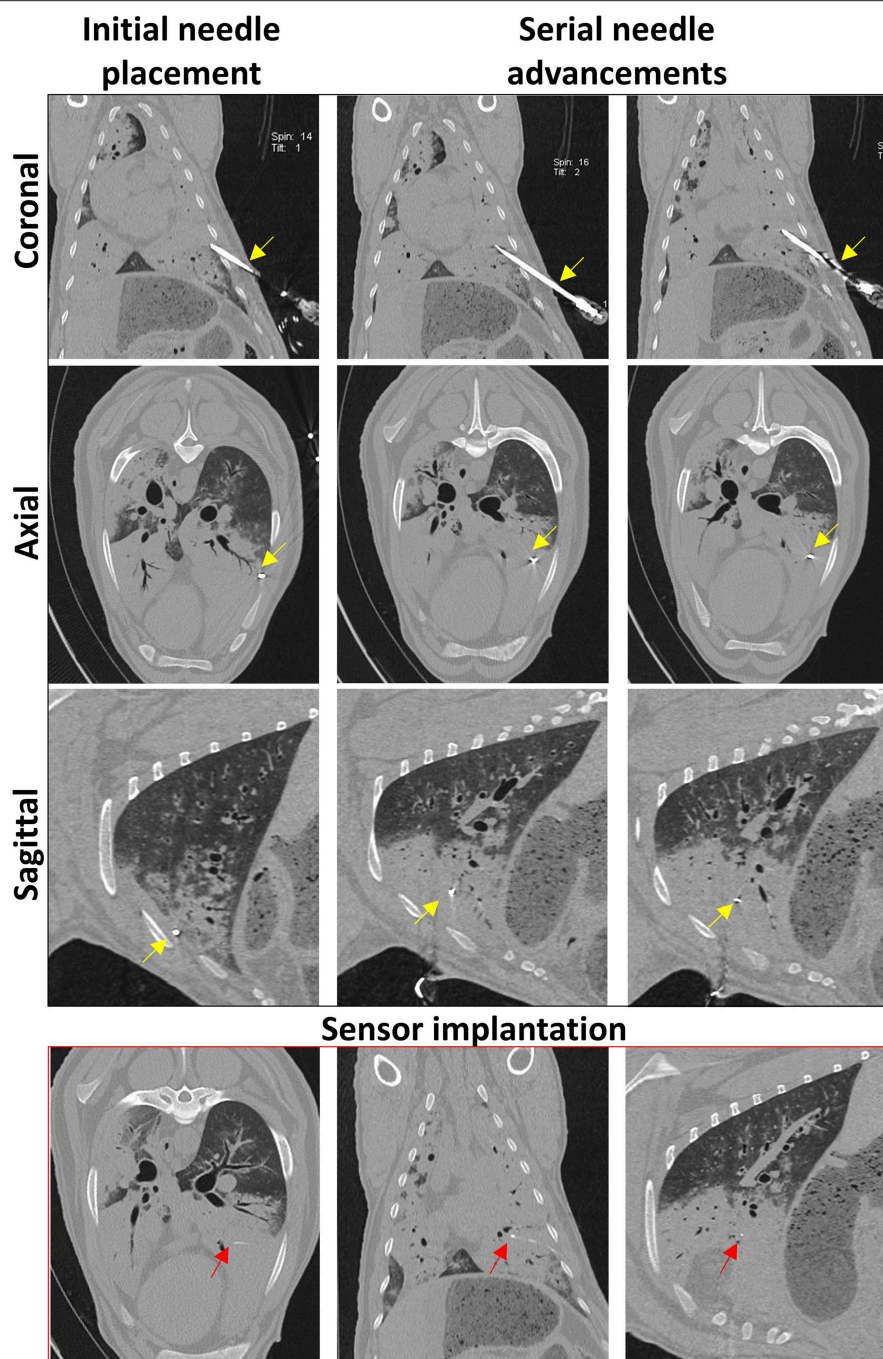


FIGURE 5 | Serial CT images taken during sensor implantation. Coronal, axial, and sagittal planes are viewed following initial needle placement and after each needle advancement. The Jamshidi needle (yellow arrows) is advanced until the tip is positioned at the desired point within the OPA lesion. CT images taken immediately post-implantation demonstrates sensor placement within OPA tissue in all 3 planes (red arrows).

RESULTS

Appropriate Anesthetic Protocols Enable OPA Sheep to Remain Physiologically Stable Throughout Anesthesia

To assess the physiological stability of OPA-affected sheep throughout anesthesia, data from blood-gas, biochemical, and

hematological analysis was combined with variables such as heart rate, respiratory rate, body temperature, and mean arterial blood pressure. Results from sheep maintained with an inspired fraction of O₂ (FiO₂) of 1.0 are shown in **Figure 6** ($n = 3-5$ per time point). The remaining 4 cases in this study were subjected to alterations in FiO₂ for sensor validation experiments and are therefore not included in this

analysis. Results showed that physiological and arterial blood variables remained stable throughout anesthesia. No statistically significant changes over time were identified in any measured variable (**Figure 6**).

Elevated blood lactate persisted throughout anesthesia but showed a tendency to reduce at later time points (**Figure 6A**). Blood pH, base excess and bicarbonate showed a similar, but opposite response (**Figures 6F,G**). Arterial O₂ partial pressure (PaO₂) showed marked individual variation (**Figure 6B**) and was consistently lower than expected given the FIO₂ of 1.0, suggesting a compromise in the degree of O₂ uptake by the alveoli. Despite this, it was possible to maintain a hemoglobin O₂ saturation (SaO₂) of $\geq 95\%$ (**Figure 6C**). Peak inspiratory pressure increased throughout anesthesia (**Figure 6D**), with mean peak inspiratory pressures at 180 min almost 1.5 times greater than that recorded at 30 min. Airway suction was frequently required to clear respiratory secretions. To support mean arterial blood pressure (**Figure 6E**), 3 sheep required management with intravenous fluids or vasopressors. Additional treatments administered during anesthesia included atropine (1 sheep; severe bradycardia), sodium bicarbonate (1 sheep; acidosis), and glucose (1 sheep; hypoglycaemia).

CT-Guided Trans-thoracic Percutaneous Sensor Implantation Resulted in a High Success Rate of Delivery of Sensors Into OPA Lesions

A total of 9 sheep underwent general anesthesia and sensor implantation (2 additional cases were excluded from analysis due to a lack of histological evidence of OPA following post-mortem examination). Of the 9 OPA-affected sheep that underwent CT-guided sensor implantations into tumor tissue, 7 cases received a single sensor implantation and 2 cases received 2 sensors implanted into a single large OPA lesion. In the case of single sensor implantations, time taken from the initial CT scan to sensor placement was 45 ± 5 min (mean $\pm$ SEM). Double implantations took a little longer, with implant times of 50 and 73 min for each case. The number of sequential CT scans and needle advancements required from the initial needle placement to obtaining the desired position within OPA tissue ranged from 3 to 5, with 4 advancements required in 9 of the 11 sensor implantations. All implantation procedures resulted in sensor placement within OPA tissue (**Figure 7**). No immediate complications were identified in 5 of the cases (**Table 2**). Estimates of the amount of radiation received by each sheep undergoing CT-guided sensor implantation were calculated. DLPs for individual topograms and full thoracic scans were 9 ± 0.3 mGy cm and 392 ± 11 mGy cm, respectively (mean $\pm$ SEM), whereas total DLP for each sheep was $2,856 \pm 392$ mGy cm (mean $\pm$ SEM).

Iatrogenic Pneumothorax Is a Potential Complication Following Percutaneous Sensor Implantation

Sensor implantation in 4 cases immediately resulted in mild pneumothoraces; however, only 2 of these cases required

treatment with percutaneous thoracocentesis. CT scans post-thoracocentesis confirmed lung lobe re-expansion and removal of most of the air from within the thoracic cavity. Sensor positioning was not affected by the occurrence of a pneumothorax and the sensor remained within the OPA lesion post-thoracocentesis (**Figure 8**).

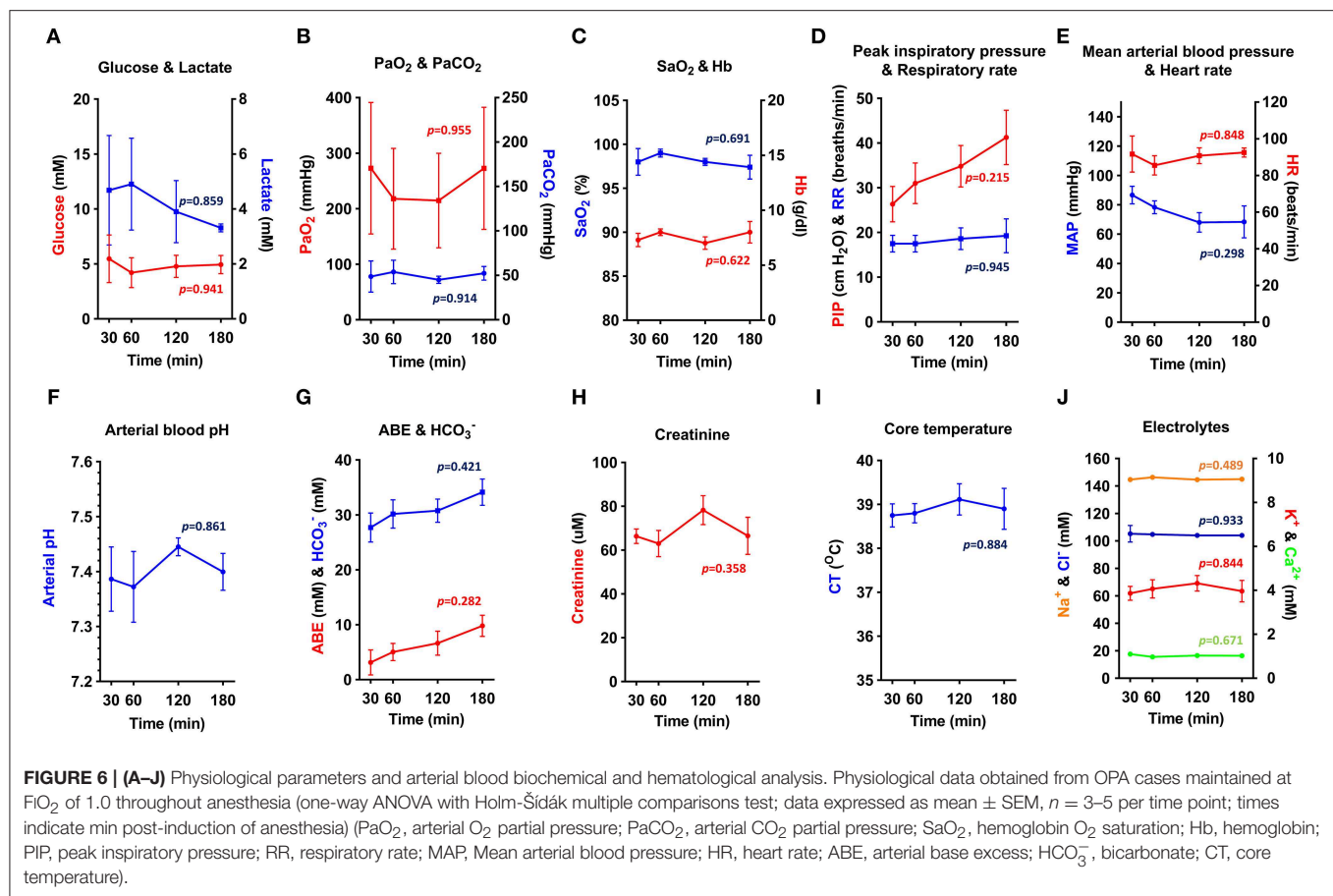
All Implantation Sites Were Identified During Post-mortem Examination

All sheep underwent post-mortem examination following euthanasia. Gross pathology allowed assessment of lung pathology, identification of the implant site, and provided the opportunity to obtain biopsy specimens for histological analysis. Gross pathology identified lesions that were in accordance with those identified on the CT scans in terms of number of lesions, location, and size. All sensor implantation sites were successfully identified with an entry site seen in the visceral pleura directly overlying OPA tissue. In 1 case an area of petechial hemorrhage was evident to the lung surface in the region of the implantation site; however, the remaining cases had no gross evidence of parenchymal hemorrhage or haemothorax (**Figure 9**). Following examination of gross pathology, the implant site was dissected from the OPA tissue. The biopsy specimen was used for both OPA diagnosis and to assess the effects of the implantation procedure on OPA/lung tissue. Histological examination confirmed OPA diagnosis in all 9 cases (the 2 excluded cases were reported as lung consolidation with marked pleural fibrosis and pleuritis). Evidence of hemorrhage within the needle tract and erythrocytes present within tumor tissue immediately adjacent to the implant site were identified in all cases (**Figure 10**).

DISCUSSION

Similarities between OPA and human lung adenocarcinomas in terms of disease presentation, progression, and histological classification has led to the recognition of OPA as an excellent model for studying human lung cancer biology (24). *In vitro* (25–29) and *in vivo* (30–35) OPA experimental models are well-documented and have been successfully used to identify molecular pathways involved in lung cancer pathogenesis. However, for OPA to be used as a translational pre-clinical research model for human lung cancer, techniques used in the diagnosis and treatment of human patients must be incorporated into the model. In order to achieve this aim, protocols currently used in human thoracic medicine were incorporated into our novel OPA model, which was used for validation of the sensors which have been developed as part of this project (20).

Although certain thoracic procedures in human medicine are commonly performed under local anesthesia, general anesthesia was mandatory in our OPA model to ensure animal and personnel safety. It is therefore important to consider the general anesthetic requirements of these sheep if they are to be used in translational research. General anesthesia of sheep with OPA can be challenging but is entirely feasible if facilities and expertise are in place to provide, if required, respiratory, and cardiovascular support. These animals have variable amounts



of respiratory compromise resulting from the OPA lesion(s), lung lobe consolidation, increased respiratory tract secretions, secondary infections, and anesthesia-induced atelectasis. All these factors will hinder the effective movement of inspired O₂ into the blood, leading to lower PaO₂ levels. Adequate blood O₂ content in our cases was maintained by increasing the inspired fraction of O₂ in combination with mechanical ventilation. The use of elevated peak inspiratory pressures in our cases was well-tolerated and was necessary to achieve adequate ventilation due to reduced compliance of the diseased lungs. The sheep in our study also had elevated blood lactate levels, which could have been caused by global tissue hypoxia; however, as neoplasia itself can elevate blood lactate levels (36) it is difficult to know its specific underlying etiology. The decrease in lactate levels that occurred throughout anesthesia may have been due to the provision of intravenous fluid therapy and mechanical ventilation which contribute to improved tissue O₂ delivery. Although we have shown that OPA cases may require additional anesthetic monitoring with respiratory and cardiovascular support, all our cases were successfully managed throughout sensor validation experiments. These results provide evidence that OPA cases, even with relatively large tumors (as was seen with a number of our cases), can be used in procedures that require general anesthesia. Although, it should be noted that these were pre-clinical OPA cases identified by

ultrasound screening and sheep showing clinical signs of OPA were specifically excluded from the study.

Lung cancer diagnosis in human patients is performed through immunohistochemistry using aspirates or biopsy samples taken using a flexible bronchoscope (37), or via a minimally-invasive trans-thoracic approach (38–40). The choice of which technique to use is dependent on the location of the lesion. Central lesions involving a bronchus will be readily assessable with a bronchoscope, whereas peripheral lesions that are either not visible on endobronchial examination (41), <3 cm in diameter or those that do not show a bronchus entering the lesion on CT images will be more suited to minimally invasive trans-thoracic needle biopsy (TTNB) (38, 39, 42). Both endoscopic and percutaneous biopsy techniques could have been modified for use in our OPA model; however, for several reasons the trans-thoracic percutaneous approach was chosen. Naturally-occurring JSRV infection and transformation will typically result in OPA lesions forming initially at peripheral lung lobe regions. It is only as neoplastic foci enlarge and coalesce that central lobe regions become affected. Although tumor tissue will frequently involve bronchioles, larger bronchi may remain largely unaffected. Although endoscopy can be routinely performed in sheep (43), successful endoscopic sensor implantation would only be possible in tumors which involved bronchi of sufficient diameter that could accommodate an

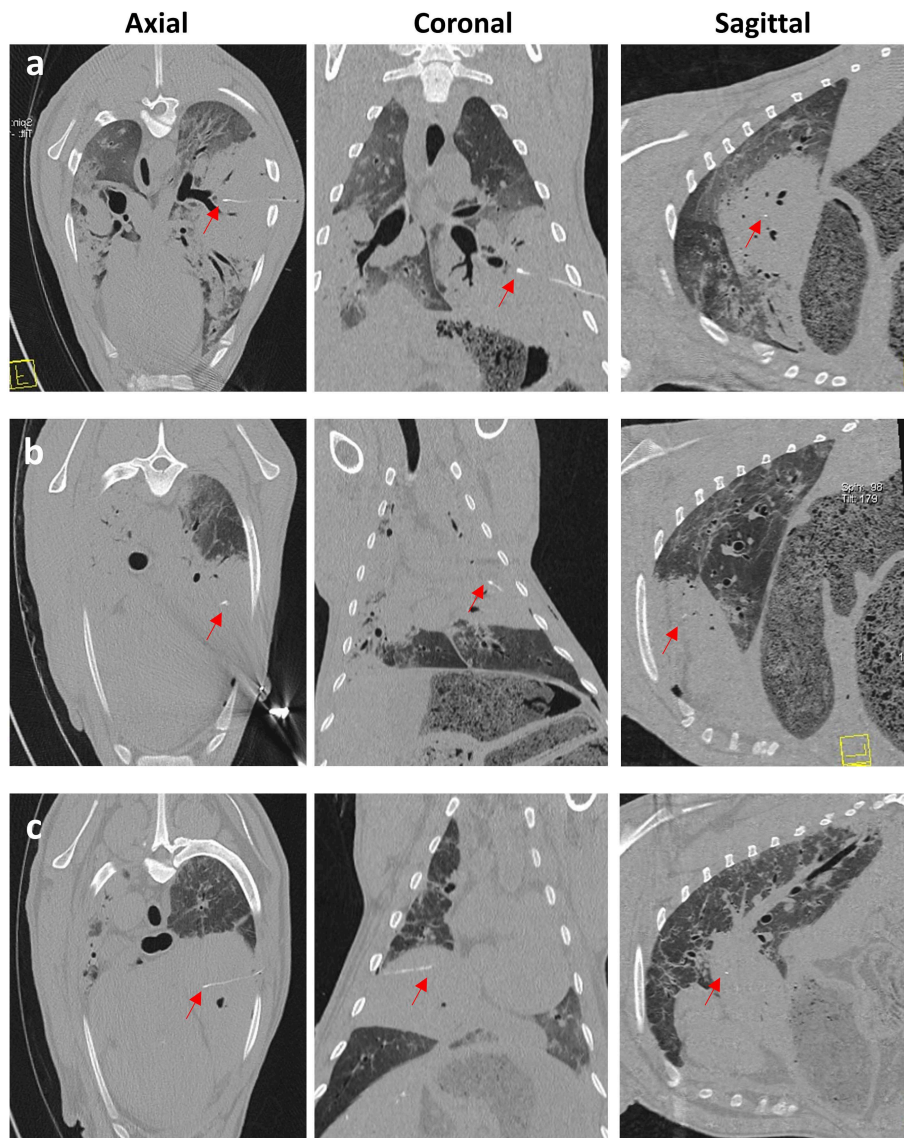


FIGURE 7 | CT images taken immediately post-sensor implantation. Images (a–c) are from 3 separate OPA cases. The sensor and lead wire (red arrows) can be seen within the area of increased radiopacity, consistent with a large OPA tumor.

endoscope. This specific set of selection criteria would limit the number of cases that could be used, and logistically could only be assessed once a sheep is anesthetized and CT images have been obtained. As OPA tumors can be associated with significant volumes of lung fluid production, present in the large and small airways, this would hamper endoscopic airway visualization and make sensor implantation extremely challenging. The sensor for which we developed the model is currently wired therefore endoscopic implantation would require the lead wire to run up through the large airways and out through the larynx to be connected to external instrumentation; the presence of the endotracheal tube would make this almost impossible. These limitations associated with endoscopic sensor delivery led us to develop

the minimally invasive trans-thoracic percutaneous approach for sensor implantation.

In human medicine TTNB requires the use of image guidance. Fluoroscopy, once the preferred imaging choice, enables needle advancements to be visualized in real-time (44); however, the technique has become less popular as it is not compatible with accessing deep lesions and the avoidance of vascular structures and bullae (45). Image guidance using ultrasound enables needle movements to be monitored precisely and quickly during the TTNB (46, 47); however, its use is restricted to peripheral lesions that produce an acoustic window. CT is currently the most commonly used image guidance technique for TTNB (48–50). Unlike fluoroscopy, CT allows accurate planning of needle path trajectories that avoid aerated lung,

TABLE 2 | Details of OPA cases used and implantation results.

Case	Signalment	Weight (kg)	CT lesion localization	No. of needle advancements	Time required for implantation (min)	Complications post-sensor placement
1	Highlander Female Adult	65	Left caudal lobe: 1 focal lesion at cranial aspect of caudal lobe, ~4–5 cm diameter	4	31	Mild pneumothorax: not treated
2	Blackface Female Adult	51	Right caudal lobe: 1 diffuse area at caudal aspect ~4–5 cm diameter	3	58	Mild pneumothorax: treated successfully with a single thoracocentesis
3	Blackface Female Adult	39	Left caudal lobe: Entire lobe affected	4	46	None
4	Blackface Female Adult	52	Left cranial lobe: Entire lobe affected	4	56	None
5	Blackface Female Adult	57	Right cranial lobe: Almost entire lobe affected	4	26	None
6	Blackface Female Adult	39	Left caudal lobe: 1 Focal lesion at caudal aspect of caudal lobe, ~10–15 cm diameter Left cranial lobe: Entire lobe affected	4	60	Mild pneumothorax: not treated
7	Blackface Female Adult	55	Right cranial lobe: Entire lobe affected Left cranial lobe: Entire lobe affected, extending into the cranial aspect of the left caudal lobe	5	43	None
8	Blackface Female Adult	58	Right accessory lobe: Entire lobe affected Left cranial lobe: Entire lobe affected, extending into the cranial aspect of the left caudal lobe	4	73	None
9	Mule Female Adult	64	Right cranial lobe: Almost entire lobe affected	4	50	Mild pneumothorax: treated successfully with a single thoracocentesis

Cases 1–7 had single sensor implantations, whereas cases 8 and 9 had 2 sensors implanted. Signalment, CT localization, time from initial CT scan to sensor implantation, number of CT scans/needle advancements required, and immediate post-implantation complications are provided.

bullae, fissures, and blood vessels. The procedure can also be used to sample central lesions, lesions <1 cm in diameter (51) and, similar to ultrasound, can distinguish between necrotic and solid regions of a lesion, allowing for more accurate needle positioning and better diagnostic samples to be obtained. CT can be combined with fluoroscopy (CTF) to allow needle adjustments to be made in almost real-time. The technique is primarily used for very small lesions located in difficult to access thoracic regions (costodiaphragmatic recess, near to the mediastinum or critical at-risk structures) and can be performed quickly, which is advantageous in un-cooperative or high-risk patients (52). Although any of these image techniques can be integrated for use within the OPA model, CT was chosen in our study for several reasons. CTF was not considered necessary as sheep were selected for use in our experiments on the

basis that they had reasonably large OPA lesions in relatively accessible lung regions. CTF would also have required the use of lead aprons and radiation shields for safety purposes. Although ultrasound guidance could have been used for sensor implantations into OPA lesions affecting pleural surfaces, the technique could not provide an assessment of pathological lesions occurring throughout the entire thorax, and thus cannot be used to aid the selection of the most appropriate lesion for implantation. These factors directed us to use CT guidance for sensor implantations.

Serial CT scans were performed during the implantation procedure, with each CT scan reviewed at each stage of the process. The initial CT scan was used to select the OPA lesion to undergo sensor implantation, while sequential scans were used to assess needle trajectory and position. Lesion selection

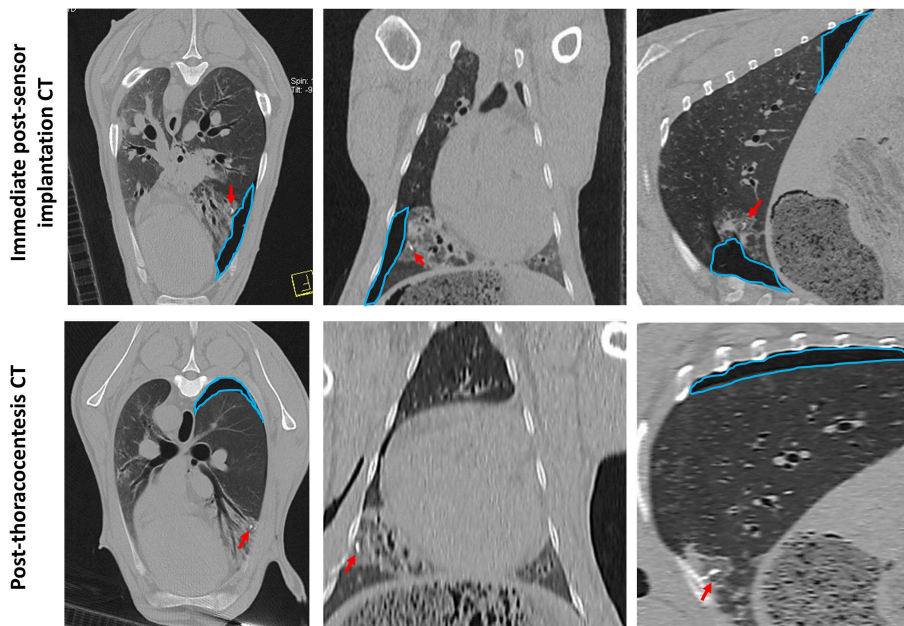


FIGURE 8 | CT images documenting iatrogenic pneumothorax. The OPA target lesion is seen as a patchy area of increased radiopacity affecting the ventral region of the left caudal lung lobe. CT scans were taken immediately post-sensor implantation and following thoracocentesis. A mild pneumothorax (outlined in blue) was evident immediately post-sensor implantation, predominately localized to the region of the implantation site. The sensor can be seen within OPA tissue (red arrows). Thoracocentesis was performed immediately post-sensor placement, which resulted in lung lobe re-expansion and removal of most of the air from within the thoracic cavity; however, a small pneumothorax at the dorsal aspect of the thoracic cavity was still evident (outlined in blue).

and needle path planning was based on known risk factors associated with the development of TTNB complications in human patients, predominantly pneumothorax and hemorrhage. Small lesions and the presence of emphysema (53) or chronic obstructive pulmonary disease (54) can increase complication rates. Although it is possible that these diseases can occur with OPA, we did not see any evidence of them based on CT imaging; therefore, their association with complications seen in the OPA model is likely to be low. Technical factors associated with performing TTNB are also known to influence the occurrence of post-operative complications, these factors are likely to have played a more significant role in the complication rate seen in our model. Technical factors that can increase the risk of TTNB complications include increased amounts of normal aerated lung crossed by the needle (55, 56), a small oblique needle angle with the thoracic pleura (57), repositioning the needle multiple times (58), a greater number of sampling procedures (59), the absence of previous ipsilateral surgery (60), using a trans-fissure approach (56) and damage to thoracic vasculature. In accordance with these known risk factors, lesions were chosen so that the needle path avoided passing through bullae, large blood vessels, bronchi and interlobar fissures. If more than one lesion was present, a peripheral lesion was chosen to decrease the amount of lung tissue that would be traversed (61). It is interesting to note that procedural length or needle dwell time within the lung is not associated with increased risk of pneumothorax (59). In our model single sensor implantations were performed in a time of 45

± 5 min (mean $\pm$ SEM), similar to studies in human patients that document CT-guided TTNB times of up to 66 min (59).

In human medicine, monitoring and standardization of radiation dose from diagnostic and interventional procedures is now commonly performed in an effort to minimize potential risks to patients from radiation exposure (23). In our model the mean DLP for a single full thoracic scan was 392 ± 11 mGy cm (mean $\pm$ SEM), which is comparable with recommended diagnostic reference levels of 517 mGy cm used for human thoracic CT scans (62). As previously described, multiple CT scans were performed during sensor implantations, which resulted in a total mean DLP of $2,865 \pm 392$ mGy cm (mean $\pm$ SEM). This value is higher than that reported in the human literature for patients undergoing CT-guided TTNB, with one study documenting a total mean DLP of 801 mGy cm (63). However, in the same study DLPs as high as 3,684 mGy cm were reported for patients undergoing thoracic drainage procedures. The relatively high total DLPs observed in our study was due to the need to obtain high quality images for accurate needle path planning and sensor placement, which in combination with additional post-sensor implantation scans, will have increased the total radiation dose received by each sheep. However, as our results have shown that single thoracic scans were lower than human recommended diagnostic reference levels and reports for patients undergoing thoracic drainage, this provides evidence that CT-guided techniques in sheep are comparable with similar human procedures.

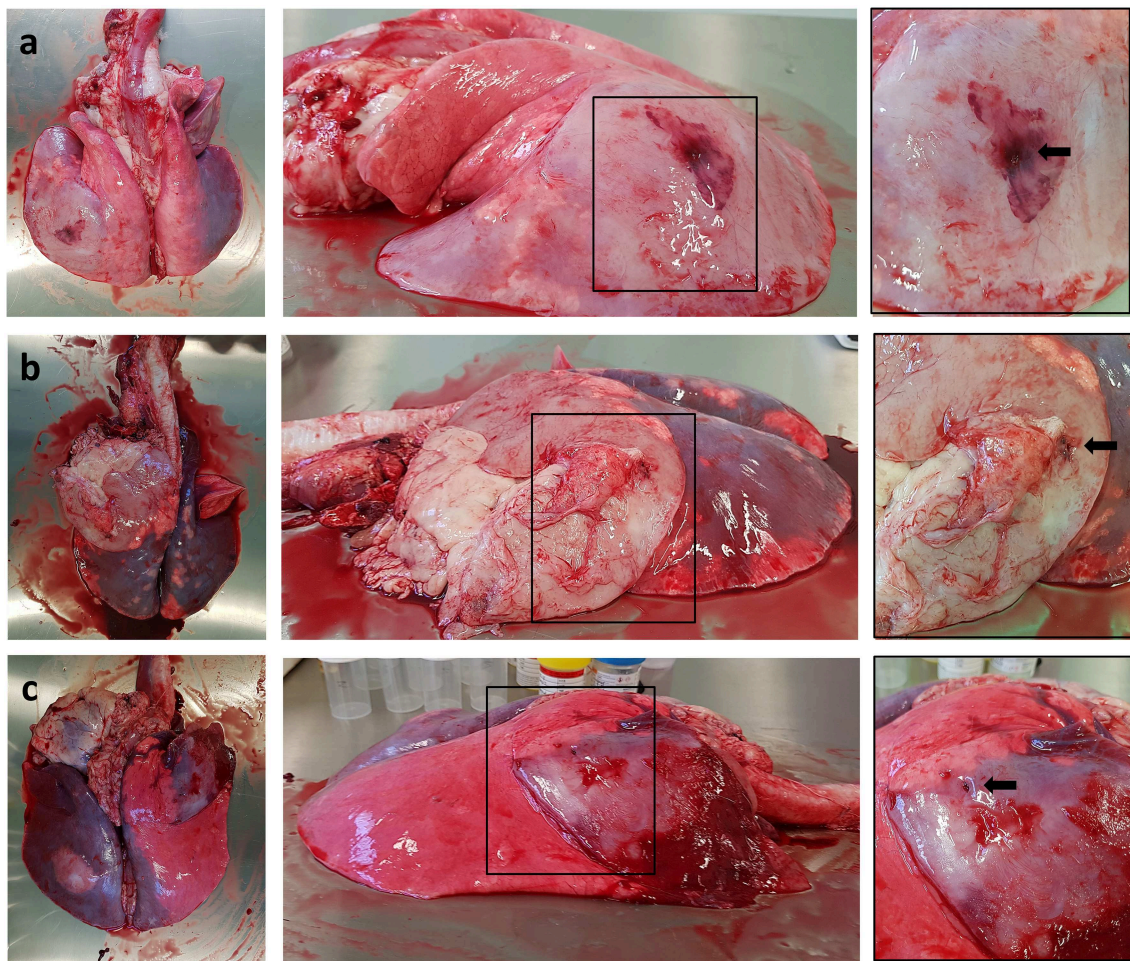


FIGURE 9 | Photographs depicting gross pathology seen in typical OPA cases. Each implantation site is highlighted with a black arrow. **(a)** Large gray consolidated mass affecting the majority of the left caudal lung lobe. An area of petechial hemorrhage can be seen on the surface of the lung surrounding the needle entry point. **(b)** Large gray consolidated mass affecting the majority of the left cranial lung lobe. Fibrous tissue can be seen adherent to the lung surface just cranial and ventral to the needle entry point. **(c)** One large dark colored mass affecting the right cranial lung lobe containing the implant site, with a further focal lesion within the left caudal lung lobe.

Following the implantation procedure, a CT scan was performed to assess for complications and to evaluate final sensor positioning. Although numerous TTNB associated complications have been documented which include infection, air embolism, lung lobe torsion, and needle tract metastasis, by far the most common complications are pneumothorax and hemorrhage (64).

Although pneumothorax rates as high as 54% have been documented (65, 66), accepted occurrence rates are more likely to be in the region 17–26%, of which ~14% will require percutaneous aspiration or chest tube insertion (57, 60, 67, 68). In our series of experiments, 4 out of 9 cases (44%) developed a mild immediate post-implantation pneumothorax, however only 2 required percutaneous needle thoracocentesis. The cases that received thoracocentesis resulted in lung lobe expansion, with the sensor remaining within the OPA lesion. In each case the pneumothorax did not reoccur; this was likely due to the removal

of excess pleural air from around the implant site, allowing apposition of visceral and parietal pleural surfaces. Measures that were used to reduce the risk of pneumothorax included the use of a coaxial needle, to allow the sensor to be placed with a single pleural puncture, and careful needle path planning.

Although hemorrhage is the second most common TTNB complication that occurs in ~4–10% of cases (67, 69), the development of a haemothorax is <1% (64). Bleeding may be identified through blood coming up through the bore of the needle, or as a ground-glass appearance on CT images typically in the region of the biopsy or along the needle path. Measures that were used to reduce the risk of hemorrhage included the avoidance of large pulmonary and cardiovascular vessels. Placing the needle through the center of the intercostal space also reduced the risk of damage to intercostal neurovascular bundles. In our study no cases were identified as having post-implantation hemorrhage based on CT image evaluation;

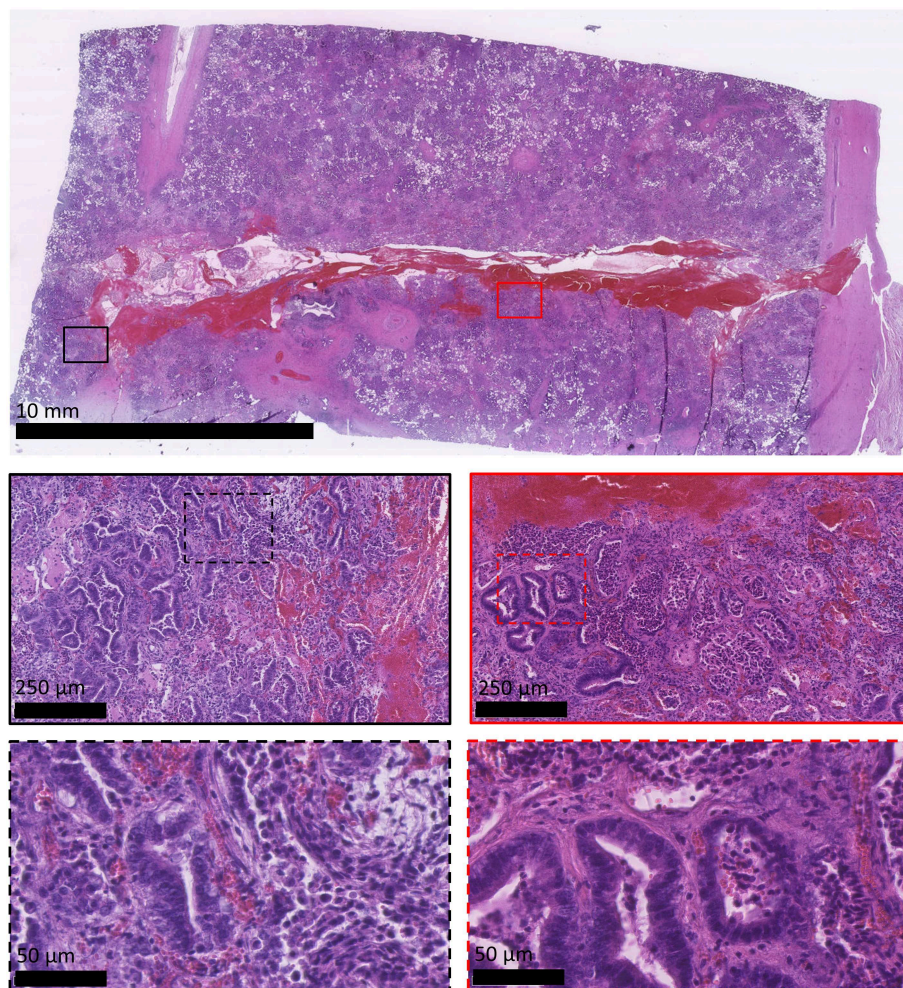


FIGURE 10 | Histological appearance of OPA sensor implantation site. All images are haematoxylin and eosin stained sections. OPA tissue including the implantation site was obtained during the post-mortem examination. The top image shows the needle tract penetrating through the visceral pleura (right-hand side of image) and into the OPA tissue. Large numbers of erythrocytes can be seen within the needle tract. Higher magnification images document erythrocytes extending up to 250 μm from the implant site, predominately within stromal tissue.

however, following post-mortem examination and implant site histopathology erythrocytes were identified within the needle tract in all cases. This finding is not unexpected as tumor tissue can have an extensive blood supply. Passage of a large needle through tumor parenchyma will inevitably damage intra-tumoural macro and microvessels. This situation is also likely to occur in human patients undergoing TTNB, but as the needle tract itself would never be biopsied there is no available data to support this. Limited amounts of erythrocytes were identified in OPA tissue away from the needle tract itself. It is possible that the OPA tissue immediately adjacent to the implant site and needle tract has reduced compliance compared with normal lung tissue, and could potentially act as a tamponade, preventing the escape of erythrocytes into the tumor tissue. The under reporting of alveolar hemorrhage based on the CT images is likely due to the appearance of the OPA lesions themselves. All cases had large OPA lesions involving the visceral pleura that were characterized

as having increased radiopacity, frequently with the presence of air bronchograms. This CT appearance of typical OPA tumors would have likely obscured any hemorrhage that occurred within the needle tract itself.

The large needle size required for sensor implantation probably contributed to the complication rate encountered in the model. The needle diameter had to exceed that of the IMPACT sensor and lead wire. Consequently, an 8G Jamshidi needle was selected, which is considerably larger than the 18–22G needles that would be routinely used in human TTNB procedures. Ongoing development and miniaturization of the sensors will allow smaller diameter needles to be used. Although smaller diameter needles may reduce the occurrence of complications, our current model has shown a comparable complication rate to that seen in human patients undergoing TTNB.

Although not considered within this article, the primary aim of developing this novel OPA model was to validate the *in vivo*

functionality of the IMPACT O₂ and pH sensors within a tumor microenvironment. In order to achieve this goal, we performed a series of physiological challenges following sensor implantation. These challenges included altering blood oxygenation levels through FiO₂ manipulations and varying blood pH levels through administering inhaled CO₂ and altering ventilation rates. The results of sensor validation experiments will be published in separate articles and will highlight the ability of the OPA model to be used to validate novel medical technologies.

CONCLUSION

This paper has described the use of naturally-occurring pre-clinical OPA cases in the development of a novel *in vivo* ovine model for the CT-guided trans-thoracic percutaneous implantation of sensors into OPA tumors. This is, to the best of our knowledge, the first description of the use of naturally-occurring OPA cases as a surgical model. Through the integration of techniques such as ultrasound, general anesthesia, CT and surgery into the OPA model, we have demonstrated its translational potential and effectiveness as a pre-clinical research tool for human lung cancer. We have also shown our model to be comparable to TTNB in human patients in terms of procedure duration, radiation exposure, and complication rate. We believe this model can be developed further for other pre-clinical uses, such as the procurement of biopsy specimens, the development of medical devices for the local delivery of chemotherapeutic agents, monitoring the tumor microenvironment and in the assessment of the effectiveness of RT or systemic chemotherapeutic agents. This model has great potential to not only advance the molecular understanding of human lung cancer, but to also improve pre-clinical research and enhance the treatment of human lung cancer patients.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

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ETHICS STATEMENT

Studies were undertaken under a UK Home Office Project License in accordance with the Animals (Scientific Procedures) Act 1986 and with approval from the University of Edinburgh Animal Welfare and Ethical Review Boards. The recommended guidelines for welfare and use of animals in research were followed.

AUTHOR CONTRIBUTIONS

DA and AM secured funding for this research and conceptualized the initial work. MG with contributions from PS developed the surgical procedure. SG, RC, and RG conducted all anesthetic procedures. JRKM performed all engineering work. MG wrote the majority of the manuscript and composed the figures, with contributions from JM and SG who wrote parts of the introduction and anesthesia sections respectively. CC and DG were involved with obtaining pre-clinical OPA cases. Critical revisions were made by all authors. All authors read and approved the final manuscript.

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Immunohistochemical Characterization of Procaspace-3 Overexpression as a Druggable Target With PAC-1, a Procaspace-3 Activator, in Canine and Human Brain Cancers

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Gliomas and meningiomas are the most common brain neoplasms affecting both humans and canines, and identifying druggable targets conserved across multiple brain cancer histologies and comparative species could broadly improve treatment outcomes. While satisfactory cure rates for low grade, non-invasive brain cancers are achievable with conventional therapies including surgery and radiation, the management of non-resectable or recurrent brain tumors remains problematic and necessitates the discovery of novel therapies that could be accelerated through a comparative approach, such as the inclusion of pet dogs with naturally-occurring brain cancers. Evidence supports procaspase-3 as a druggable brain cancer target with PAC-1, a pro-apoptotic, small molecule activator of procaspase-3 that crosses the blood-brain barrier. Procaspace-3 is frequently overexpressed in malignantly transformed tissues and provides a preferential target for inducing cancer cell apoptosis. While preliminary evidence supports procaspase-3 as a viable target in preclinical models, with PAC-1 demonstrating activity in rodent models and dogs with spontaneous brain tumors, the broader applicability of procaspase-3 as a target in human brain cancers, as well as the comparability of procaspase-3 expressions between differing species, requires further investigation. As such, a large-scale validation of procaspase-3 as a druggable target was undertaken across 651 human and canine brain tumors. Relative to normal brain tissues, procaspase-3 was overexpressed in histologically diverse cancerous brain tissues, supporting procaspase-3 as a broad and conserved therapeutic target. Additionally, procaspase-3 expressing glioma and meningioma cell lines were sensitive

to the apoptotic effects of PAC-1 at biologically relevant exposures achievable in cancer patients. Importantly, the clinical relevance of procaspase-3 as a potential prognostic variable was demonstrated in human astrocytomas of variable histologic grades and associated clinical outcomes, whereby tumoral procaspase-3 expression was negatively correlated with survival; findings which suggest that PAC-1 might provide the greatest benefit for patients with the most guarded prognoses.

Keywords: glioma, meningioma, brain cancer, procaspase-3, PAC-1, canine comparative

INTRODUCTION

In 2018, approximately 23,800 adults and 3,560 children in the US were diagnosed with malignant primary brain or spinal cord tumors, and soberingly, 16,830 adult deaths were attributed to inadequate treatment of these primary CNS tumors (1). Approximately 75% of aggressive brain cancers in humans are classified as malignant gliomas and the prognosis for patients with either anaplastic astrocytoma (grade III) or glioblastoma multiforme (GBM; grade IV) is poor due to the invasive nature of these neoplasms. Even with multimodality therapies including surgery and radiochemotherapy, median survival times for patients diagnosed with anaplastic astrocytoma or GBM are less than 36 or 15 months, respectively (2).

Paralleling the aggressive disease course of invasive malignant gliomas, higher grade meningiomas (WHO grades II and III) referred to as atypical and anaplastic, respectively, remain clinically problematic in a subset of human patients. For atypical and anaplastic meningiomas, the likelihood of local recurrence is 29–52% and 50–94%, respectively (3), and is driven by the brain invasive characteristics of these higher grade meningiomas (4–7). Of exceptional gravity are the outcomes for patients diagnosed with anaplastic meningiomas, where the average 5-year survival rates range from 30 to 60% (8, 9).

Since there have been few improvements in the treatment of malignant glial tumors and invasive meningiomas over the past decade, the discovery of new treatments for malignant CNS tumors are needed to improve long term outcomes in these affected patient populations. To identify and validate druggable targets and novel treatment strategies, the inclusion of model systems that most faithfully recapitulate the natural course of brain cancer initiation, promotion, and progression should be included into the therapeutic development path. Collectively, the scientific community has utilized diverse experimental systems and comparative models to advance the study of CNS malignancies, including dogs with spontaneously-arising brain cancer (10–13).

Similar to humans, primary brain tumors are a significant cause of morbidity and mortality in dogs, affecting up to 4.5% of the aged population (14). The most common CNS brain tumors in dogs are meningiomas (~45–50%), gliomas (~40–70%), and choroid plexus neoplasms (~5–7%) (15–18). Treatment options for dogs with brain cancer include surgery, radiation therapy, chemotherapy, or a combination of modalities (19–23). Since the tumor incidence, tumor histologies, and molecular genetic features of canine brain tumors are remarkably similar to their

human counterparts, this positions dogs uniquely as translational models for human brain cancer biology and investigational therapeutic research (15, 16, 24–27).

In both humans and companion animals, there is a need to identify effective treatment modalities for brain tumors, especially those that are difficult to resect, with the aims to significantly improve quality of life and survival times. In the age of personalized medicine, ideal therapeutics should target molecular aberrations within the cancer cell population, while sparing normal tissues from potentially harmful side effects. In both the human and veterinary literature, evasion of apoptosis is a common cellular transformation in intracranial neoplasms (28–33). As such, treatment strategies that selectively activate programmed cell death in CNS tumor cells have potential to improve long term outcomes in patients diagnosed with malignant brain cancers.

PAC-1 is a blood-brain barrier (BBB) penetrant, small molecule, pro-apoptotic activator of procaspase-3 (PC-3), that possesses favorable pharmacokinetics, tolerability, and synergistic activities when combined with conventional treatment modalities in animal models of glioma, including naturally-occurring brain cancer in pet dogs (34). Mechanistically, PAC-1 activates PC-3 *in vitro* and in cancer cells through the chelation of inhibitory zinc (35, 36), and based upon PAC-1's binding affinity for zinc ($K_d \sim 40$ nM), selective chelation of labile zinc from PC-3 is achieved in the absence of disrupting the function of proteins containing essential zinc ions (37). Importantly, cellular sensitivity to apoptosis induction with PAC-1 is associated with the resting cellular PC-3 concentration, and given many malignantly transformed cells have elevated PC-3 levels, PAC-1 therapy allows selective induction of apoptosis of cancerous cells (38). While PAC-1 has demonstrated promising preclinical activity in murine and canine models of glioma (34), whether PC-3 is robustly overexpressed and a conserved therapeutic target in naturally-occurring human and canine brain cancer malignancies has not been systematically evaluated. To address this gap in current knowledge, we sought to investigate PAC-1's broader applicability for the treatment of various brain cancer malignancies, as well as to justify the inclusion of pet dogs as a comparative tumor model for PC-3 activating strategies, we performed large-scale validation of PC-3 as a druggable target across 651 human and canine brain tumor samples, evaluated the prognostic significance of PC-3 expressions in human glial tumors, and tested sensitivity of immortalized glioma cell lines to PAC-1 under biologically achievable conditions.

MATERIALS AND METHODS

Cell Lines

Two human glioma cell lines, U118-MG and U87-MG from ATCC (Manassas, VA), and IOMM and KT21 human meningioma cell lines were provided by Gregory Riggins (Johns Hopkins University). Three canine glioma cell lines, SDT-3g, GO6A, and J3T-Bg, were provided by Peter Dickinson (UC Davis). Cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 IU/mL), streptomycin (100 IU/mL), and 10% fetal bovine serum with 5% CO₂. Cell cultures were maintained in subconfluent monolayers and passaged 2–3 times weekly as needed. Cell lines were tested by STR (short tandem repeat) analysis at the University of Arizona (human) and at the Flint Animal Cancer Center Cell Line Validation Core at Colorado State University (canine).

Antibodies

Antibodies and their manufacturers, isotypes, pretreatments, dilutions, and times of application are provided in **Table 1**. Antibodies were purchased from the listed manufacturer or were provided by Stéphane Lezmi.

Cell Protein Collection

Cells were grown in culture until 80–90% confluent. Cells were washed with PBS and trypsinized, then collected via centrifugation and washed again with PBS. Resultant cell pellets were homogenized with 100 µL of M-PER (Pierce, Rockford, IL), then mixed with fresh Pierce

protease inhibitor cocktail solution (diluted 1:100 for final working solution). The homogenate was placed on a shaker for 15 min at room temperature (RT). Cellular protein concentrations were determined using a standard assay kit (BCA, Pierce, Rockford, IL).

Tissue Collection

Tissues were collected from previously euthanized shelter dogs. Brains were collected within 5 h of death from 21 dogs with no prior history of clinically overt systemic illness. Sections of the cerebral cortex, hippocampus, cerebellum, and brain stem (obex) were taken from each dog; samples from each anatomic area were preserved both in 10% buffered formalin and via flash freezing with dry ice. Frozen samples were stored at –80°C until analysis. Small sections of frozen tissue were weighed and then added to an Eppendorf tube with Tissue Protein Extraction Reagent (T-PER, Pierce, Rockford, IL) at recommended concentrations and manually homogenized. The tissue sample was placed on a shaker for 15 min at RT, then centrifuged. Supernatants were collected and used to determine protein concentration using a standard assay kit (BCA, Pierce, Rockford, IL).

Western Blot Analysis

For evaluation of procaspase-3, PARP, cleaved PARP, and XIAP expressions, 50 µg samples of cells were electrophoresed on 12% polyacrylamide gel, and then transferred electrophoretically to a nitrocellulose membrane. Beta-actin (Abcam #ab6276-100, Cambridge, MA) was used as a loading control. Band intensity

TABLE 1 | Antibodies.

Antibody	Manufacturer	Host & Isotype	Pre-treatment	Dilution	Time	Staining
Procaspase-3, #ab32150	Abcam, Cambridge, MA	Rabbit monoclonal IgG	Diva Decloaker 5 min	1:3,000	30 min	Automated
Caspase-3, #ab13585	Abcam, Cambridge, MA	Mouse monoclonal IgG1	Diva Decloaker 5 min	1:100	60 min	Automated
Calbindin-D, #c9848	Sigma-Aldrich, St. Louis, MO	Mouse monoclonal IgG1	Diva Decloaker 5 min	1:3,000	30 min	Automated
Caspase-3, #ab2171	Abcam, Cambridge, MA	Mouse monoclonal IgG2a	Diva Decloaker 5 min	1:50	60 min	Automated
GAD67, #mab5406	Millipore, Billerica, MA	Mouse monoclonal IgG2a	Diva Decloaker 5 min	1:800	30 min	Automated
Doublecortin, #ab18723	Abcam, Cambridge, MA	Rabbit polyclonal	Citrate 40 min at 87°C	1:3,000	8 hr	Manual
NeuN, #ab177487	Abcam, Cambridge, MA	Rabbit monoclonal	Citrate 30 min at 97°C	1:1,000	30 min	Manual
GFAP, #Z0334	Dako, Santa Clara, CA	Rabbit monoclonal	Trypsin 10 min at 37°C	1:500	30 min	Manual
Green fluorescent antibody, A865	ThermoFisher Scientific, Waltham, MA	Goat anti-Mouse IgG APC	-	1:100	60 min	Manual
Red fluorescent antibody, A11034	ThermoFisher Scientific, Waltham, MA	Goat anti-Rabbit IgG, Alexa Fluor 488	-	1:200	60 min	Manual
Beta Actin, #ab6276-100	Abcam, Cambridge, MA	Mouse monoclonal IgG1	-	1:5,000	30 min	Manual

was measured with Image Lab computer software (v. 6.0.0, BioRad Laboratories, Hercules, CA).

Immunohistochemistry

Formalin-preserved samples were fixed for 24 h, then paraffin-embedded. Immunohistochemical (IHC) staining was performed using an indirect immunoperoxidase technique with diaminobenzidine (DAB) as the chromogen for procaspase-3 (PC-3), caspase-3 (C-3), and isotype controls (GAD67 and Calbindin-D-28K). For these antibodies, IHC staining was performed using an autostainer (intelliPATH FLX, Biocare, Concord, CA). Processed slides were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase activity was blocked with Biocare #PX968 Peroxidized 1 at RT for 5 min, rinsed with TBS wash buffer, and then incubated for 10 min at RT with Biocare #BP974 Background Punisher. Slides were incubated with procaspase-3 antibody (Abcam #ab32150) for 30 min, washed, and then incubated with Rabbit-on-Canine HRP-Polymer (Biocare #RC542) for 30 min. Slides were washed with TBS, then the reaction was developed using DAB substrate for 5 min. Slides were counterstained with Mayer's hematoxylin. Human tonsil and canine lymph node served as species-specific negative and positive controls.

Additionally, canine brain tissue with moderately intense PC-3 staining was further evaluated using double fluorescent staining, in which PC-3 or PC-3 and caspase-3 (C-3) staining was coupled with doublecortin, GFAP, NeuN, or synaptophysin. Slides were deparaffinized in xylene twice for 5 min. Slides were rehydrated with 100% ethanol, twice for 3 min, and once with 95% ethanol for 1 min. The slides were rinsed in distilled water prior to receiving pre-treatment, as outlined in **Table 1**. Slides were rinsed in PBS-Tween 20 two times for 2 min. Slides were blocked with 1% BSA and incubated with both primary antibodies for 1 h at RT. Slides were rinsed in PBS-Tween 20 twice for 3 min, incubated with secondary fluorescent antibodies in PBS for 30 min, and then rinsed in PBS-Tween 20 twice for 3 min. Slides were counterstained with DAPI for 20 min at RT, and then rinsed in PBS-Tween 20 twice for 2 min. Slides were coverslipped with anti-fade fluorescent mounting medium. Canine lymph node served as negative and positive control. Slides were imaged with a Zeiss LSM 700 confocal microscope.

Immortalized Glioma and Meningioma Cell Sensitivity to PAC-1

All cells were allowed to attach to cell culture plates at least 24 h prior to treatment. A DMSO vehicle control was used in all experiments. Cell viability was assessed using a CellTiter-Blue assay (Promega, Fitchburg, WI). For each cell line evaluated, 2000 cells were seeded in 100 μ L of complete media in 96-well plates. Cells were treated with 72-h continuous PAC-1 therapy at doses ranging 0.1–100 μ M. Following the study period, cell viability was assessed with the CellTiter-Blue reagent and readout from a fluorescent plate reader. In all cases, dose-response curves and IC₅₀ determinations were performed with Origin software (v. 10.4.12.59996, OriginLab, Northampton, MA). For the demonstration of PAC-1 mechanistic activity, immortalized glioma and meningioma cell lines were grown in 6

well plates until confluence, and then exposed to PAC-1 at various concentrations (0 [DMSO vehicle], 6.25, 12.5, 25, and 50 μ M) for 24 h prior to whole protein lysate collection and analysis by western blot for PARP and cleaved PARP.

In vivo Activity of PAC-1 for Delaying Intracranial Glioma Growth

All animal procedures were approved by the University of Illinois IACUC (Institutional Animal Care and Use Committee; protocol #15030). 8-week-old female intact C57BL/6 mice were obtained from Charles River. Mice were allowed to acclimate to their new environment at least 7 days prior to cell implantation. The day prior to surgery, mice were anesthetized using 2–3% isoflurane in an induction chamber, then were maintained on 1.5–2% continuous flow isoflurane via a nose cone. A $\sim$ 1 cm square area was shaved caudal to the orbit and just to the right of midline in preparation for surgery. A small amount of Nair hair removal cream was used to remove residual fur. On the day of surgery, media containing non-adherent GL261 neurospheres was collected. Collected GL261 cells were centrifuged at 1,500 rpm at 4°C for 5 min and viability assessed with trypan blue exclusion. GL261 cells were washed twice with Hanks Balanced Salt Solution (HBSS), then suspended in a solution of 50,000 cells/0.5 μ L and placed on ice. Mice were induced and anesthetized as previously described and a 5 mm incision was made slightly to the right of midline and just caudal to the orbit. A Stereotaxic unit was used to place the cellular implantation site +0.55 mm anterior and 2.5 mm to the right of the Bregma. The skull was punctured using a 27 g needle mounted on the Stereotaxic holder. A 0.5 μ L Hamilton syringe with a 33 g needle was advanced $\sim$ 3.5 mm ventral to the skull surface. GL261 cells were injected over a period of 1 min, and 2 min were given to allow back pressure to dissipate. The syringe was slowly raised over 30 s. The incision site was closed with a small drop of VetBond. Mice were placed in individual clean cages to allow the incision sites to heal.

Mice were imaged with MRI at days 10 and 29 following GL261 tumor implantation. Data was acquired on a vertical bore imaging scanner (Oxford Instruments, Abington, UK) equipped with a Unity/Inova console (Varian, Palo Alto, CA), operating at 14.1 T and dedicated to small animal studies. A recently patented radiofrequency coil and holder, specifically designed for mouse brain MRI/MRS, was employed to make experimental studies more informative and efficient (B. Odintsov "Tunable Radiofrequency Coil," US Patent #US 8,049,502 B2; November 1, 2011). Tumor volumes were calculated using ImageJ software. Mice received 10 days of PAC-1 ($n = 4$) or sham therapy ($n = 4$) prior to a follow-up MRI on day 29 post-implantation. Sham therapy mice received HP β CD vehicle control every 12 h via oral gavage, experimental mice received PAC-1 (100 mg/kg) in HP β CD vehicle every 12 h via oral gavage. Oral PAC-1 was administered on a 5 day treatment, 2 day off schedule.

Scoring of Immunoreactivity Data

Three commercial human microarrays (MG801a, GL2082, and GL2083a, US Biomax, Inc., Rockville, MD) and three

additional human microarrays and tissues from normal human hippocampus and medulla (courtesy Charles Eberhart, Johns Hopkins University) were evaluated for PC-3 staining intensity. The use of human tissue samples in the research conducted was approved by the Human Subjects Institutional Review Board at the Johns Hopkins University. Additionally, three canine glioma microarrays (courtesy Peter J. Dickinson, UC-Davis) and 32 archived samples from the University of Illinois Veterinary Diagnostic Laboratory were evaluated for PC-3 immunostaining using ab32150 (Abcam, Cambridge, MA). Samples were assigned a numerical designation, and a random number generator was used to select each sample prior to evaluation.

Five hundred cells—or as many as were available—from each sample were graded by one observer (LJS) on a continuous grading scale, and the percentage of negative, faintly staining, moderately staining, and strongly staining cells were recorded (see **Figure 1** for illustration). Negatively staining samples contained <10% positive cells. Cells that had <50% cytoplasmic staining were graded as “faintly stained,” those with >50% cytoplasmic staining were graded as “moderately stained,” and those with >50% cytoplasmic staining and in which nuclear detail was obscured by staining intensity were categorized as “strongly stained.” Manual grading was repeated three times for each sample to ensure consistency; although cell percentages

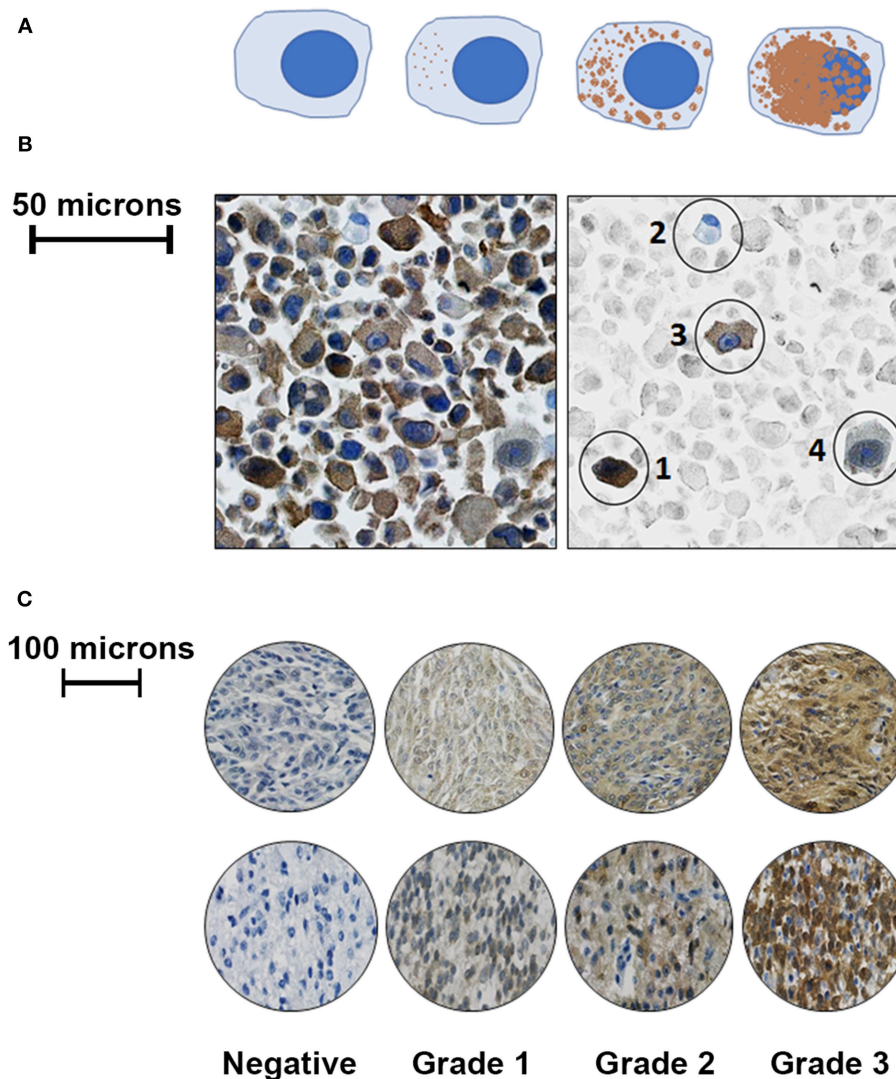


FIGURE 1 | Representative illustrations and images to demonstrate cell grading criteria. **(A)** Left to right- Negative cells contained no cytoplasmic staining for PC-3; faintly positive cells had <50% cytoplasmic staining; moderately positive cells had ≤ 50% positive cytoplasmic staining; and strongly positive cells had extensive cytoplasmic staining obscuring cellular nuclear detail. **(B)** Left: unaltered view of PC-3 immunostaining from a malignant canine astrocytoma. Right: Same image with representative cells in color, while the background cells are faded; from left to right: (1) a strongly staining cell, (2) a negatively staining cell, (3) a moderately staining cell, and (4) a faintly staining cell. **(C)**: Representative images of canine meningioma samples (top row) and astrocytoma tissue microarray cores (bottom row) that were graded following evaluation of 500 cells using the above criteria.

differed slightly between observations for the same tumor, the final manual tumor grade was the same in each case.

Microarray samples were secondarily evaluated using the iCyt automated imaging cytometer (Model TLC 1413, ThorLabs, Newton, NJ). The iCyt is a laser-scanning microscope that combines digital imaging with real time population data analysis of analytical cytometry; in addition to detecting fluorescence, the iCyt is able to detect and quantify the amount of DAB chromogen staining. Gates were set using examples of each staining category from manually graded samples. When there were multiple cores of tissue available for a single case, the mean percentages of faintly, moderately, and strongly positive cells were used to determine a final grade for the sample. Normal tissue was used to establish the threshold between negatively and positively staining tumor samples. For the canine samples, sections of normal brain were used as negative controls. For human tissues, microarray cores and tissues (courtesy Charles Eberhart, Johns Hopkins University) were used as negative controls. Raw grading scores were determined using the following formula:

$$1 \times (\% \text{ faintly positive cells}) + 2 \times (\% \text{ moderately positive cells}) + 3 \times (\% \text{ strongly positive cells})$$

PC-3 immunostaining grades were assigned based on natural numeric cutoffs observed following use of this formula, and tumors were categorized as negative for scores <10, grade 1 or faintly staining for scores <50, grade 2 or moderately staining for scores <150, or grade 3 or strongly staining for scores >150.

RESULTS

Procaspase-3 Is Expressed Predictably and at Low Levels in the Normal Human and Canine Brain

Based upon our standardized grading scheme (Figure 1), within human control tissues, there was minimal PC-3 staining in human cerebral white matter and cerebellum samples. Staining was generally mild and cytoplasmic, with occasional fine, punctate nuclear staining observed in some areas. There was more intense immunostaining in the cerebral gray matter and hippocampus than in the cerebral white matter or cerebellum (Figure 2A).

Similarly, a comparable PC-3 immunostaining pattern was observed in all normal canine brains (Figure 2A). Macroscopically, in the five anatomic regions sampled, PC-3 expression appeared strongest in the hippocampus, particularly in the dentate gyrus, and in the cerebral cortical gray matter (Figure 2B). Likewise, there was less intense immunostaining in the brain stem and cerebellum. Immunoblotting for PC-3 using flash-frozen sections of canine brain corroborated these IHC findings (Figure 2B). Although the staining pattern in these various anatomic areas was consistent among the dogs sampled, there was subtle inter-individual variability in the PC-3 immunostaining intensity. By confocal fluorescent microscopy, subcellular localization of PC-3 staining was identified to be

occasionally within neurons, and consistently in synaptic-like structures on the neuronal membranes, dendrites and axons throughout the normal canine brain (Figure 2C).

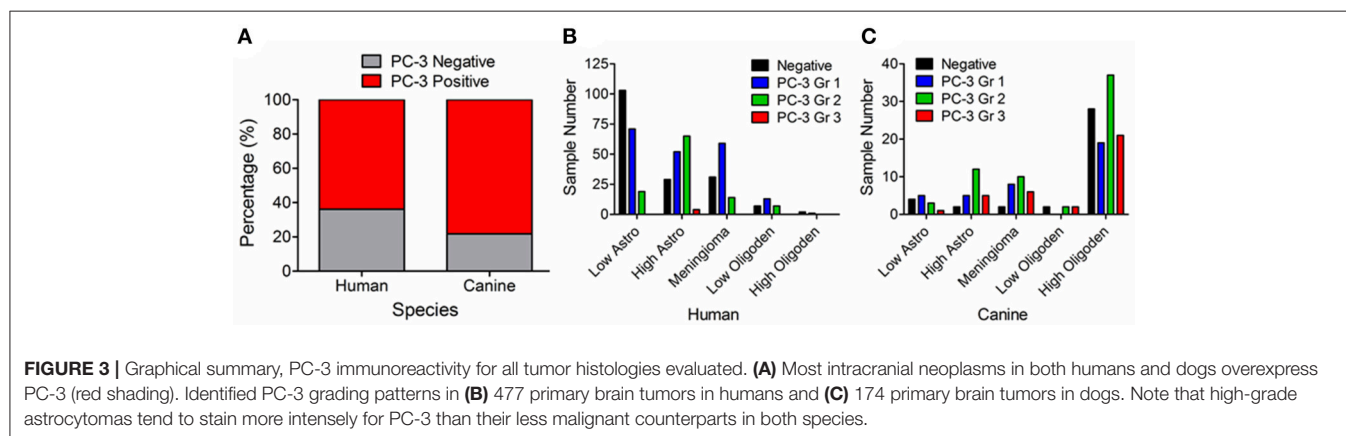
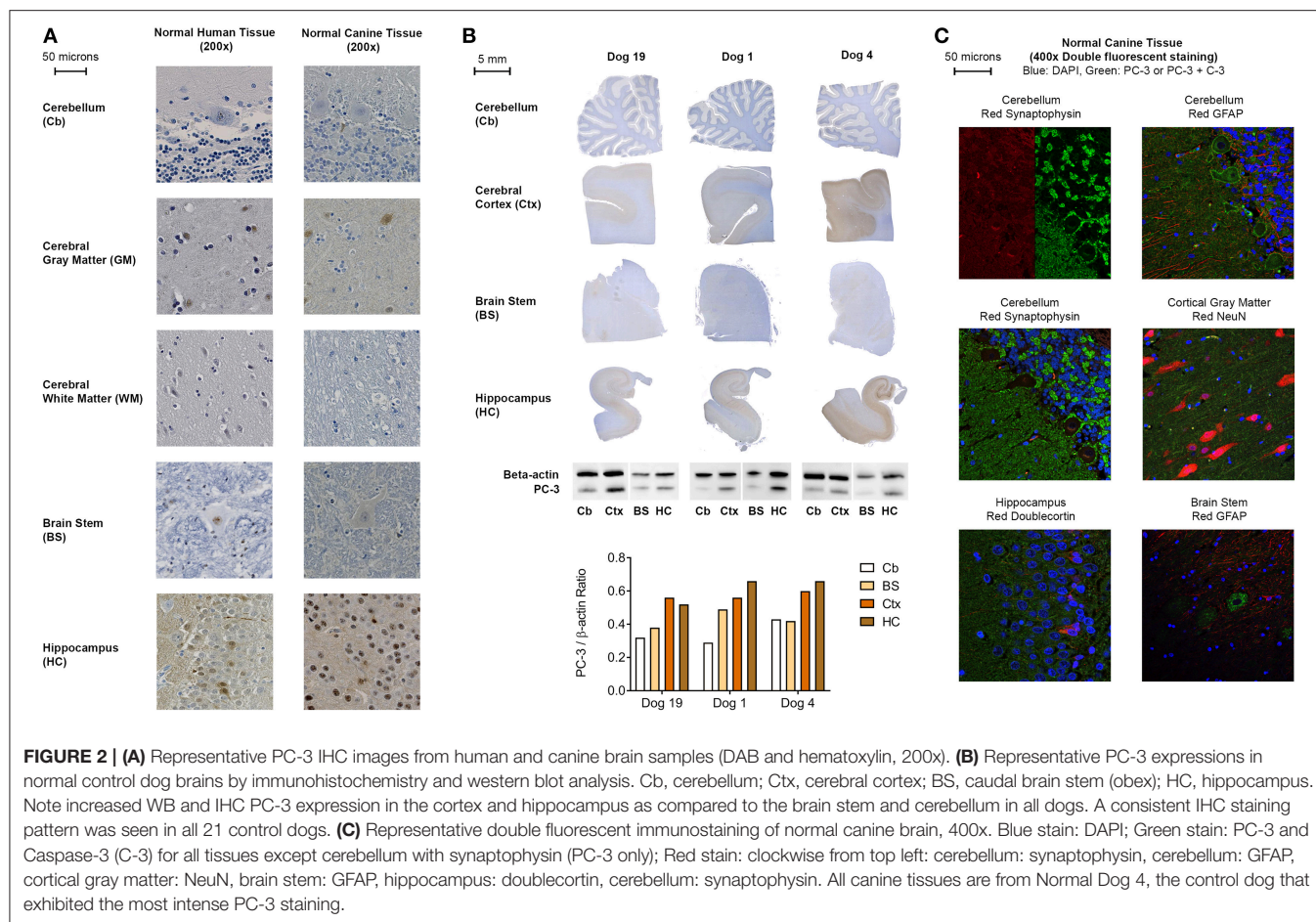
Procaspase-3 Is Overexpressed in Intracranial Neoplasms

In 477 human and 174 canine tumors, PC-3 was overexpressed based on IHC relative to normal brain tissue (Figure 3A). Specifically, 62% (211/343) of human astrocytomas and 83% (31/37) of canine astrocytomas overexpressed PC-3. Within this subset of tumors, low histologic grade astrocytomas had less intense PC-3 expression as compared to high histologic grade astrocytomas in both species (Figures 3B,C). 70% (73/104) of human meningiomas and 92% (24/26) of canine meningiomas overexpressed PC-3, while 70% (21/30) of human oligodendrogliomas and 73% (81/111) of canine oligodendrogliomas overexpressed PC-3 (Figures 3B,C, respectively). In 32 cases of primary canine brain tumors from archived cases at the University of Illinois Veterinary Diagnostic Laboratory, 87.5% (28/32) of tumors overexpressed PC-3 relative to the normal brain tissue obtained from cadaver dogs.

A Kappa statistic, which measures inter-observer variation, was >0.81 for both human and canine samples, consistent with almost perfect agreement between manual and iCyt automated cytometer tumor grading (39). For human samples, Kappa = 0.92 (95% confidence interval: 0.89–0.95), and for canine samples, Kappa = 0.88 (95% confidence interval: 0.84–0.92). For all samples considered, Kappa = 0.90 (95% confidence interval: 0.88–0.93).

Immortalized Glioma and Meningioma Cell Lines Are Sensitive to PAC-1 Therapy at Biologically-Relevant Concentrations

Western blot evaluation across human and canine brain cancer cell lines showed expression of a 32 kDa protein product consistent with PC-3 (Figure 4A). Corroborating the western blot findings, IHC-stained cell pellets derived from the same cell lines also identified robust positive staining for PC-3 in both human and dog-derived cell lines. In addition to PC-3, the expression of XIAP, a member of the inhibitor of apoptosis family proteins (IAP) that negatively regulate executioner caspases, was identified in all cell lines (Figure 4B). *In vitro*, PAC-1 induced cell death in all immortalized cell lines at biologically-relevant concentrations; Figure 4C shows representative IC₅₀ dose response curves for individual cell lines, and Table 2 shows aggregate IC₅₀ data from 72 h of continuous exposure to PAC-1 for all human and canine cell lines. There was no correlation identified between IC₅₀ values and basal expressions of PC-3, XIAP, or the ratio of PC-3/XIAP across the cell lines (data not shown). Supporting PAC-1's mechanism of action, exposures to PAC-1 for 24 h across a range of concentrations (vehicle, 6.25, 12.5, 25, and 50 μM) demonstrates consistent activation of caspase-3 activities represented by either degradation of PARP, accumulation of cleaved PARP, or the combination (Figure 4D).



PAC-1 Attenuates Growth of Murine GL261 glioma

The murine glioma cell line, GL261, robustly expresses PC-3 (Figure 5A) and is sensitive to the apoptosis inducing activities of PAC-1 at low micromolar concentrations (Figure 5B). Intracranial implantation of 50,000 GL261 neurospheres into C57BL/6 female mice generates with high penetrance variably sized gadolinium contrast enhancing tumors 10-days following

implantation (Figure 5C, left). The median and range of GL261 tumor volumes prior to treatment (day 10) were similar between sham vehicle (1.5 mm³; range 0.02–3.1 mm³) and PAC-1 (1.5 mm³; range 0.09–3.4 mm³) treated mice. Over a course of 19 days, GL261 tumors grow rapidly into large, spacing occupying lesions (Figure 5C, right); with the median GL261 tumor volumes in sham vehicle treated mice being 449.2 mm³ (range 9.1–1747.6 mm³) compared to PAC-1 treated mice being 59.5

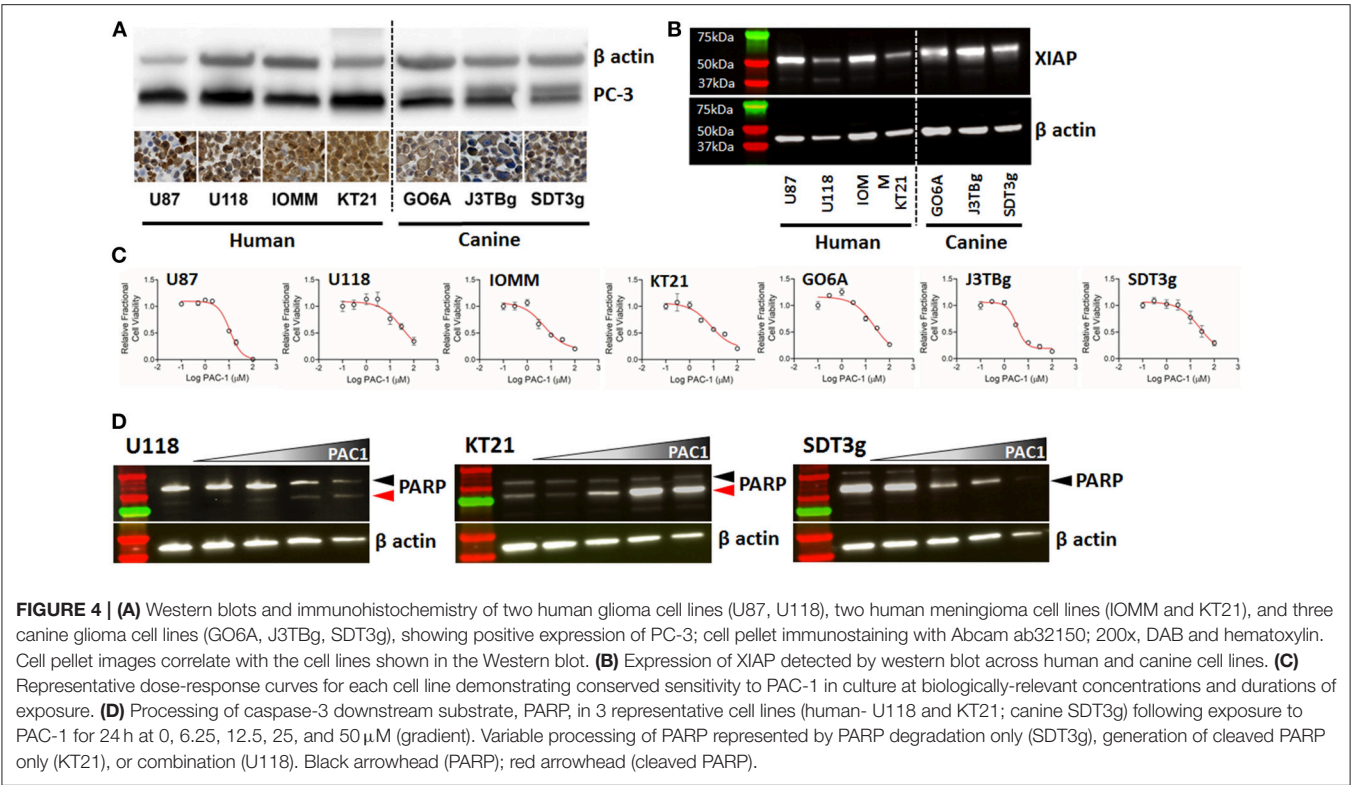


FIGURE 4 | (A) Western blots and immunohistochemistry of two human glioma cell lines (U87, U118), two human meningioma cell lines (IOMM and KT21), and three canine glioma cell lines (GO6A, J3TBg, SDT3g), showing positive expression of PC-3; cell pellet immunostaining with Abcam ab32150; 200x, DAB and hematoxylin. Cell pellet images correlate with the cell lines shown in the Western blot. (B) Expression of XIAP detected by western blot across human and canine cell lines. (C) Representative dose-response curves for each cell line demonstrating conserved sensitivity to PAC-1 in culture at biologically-relevant concentrations and durations of exposure. (D) Processing of caspase-3 downstream substrate, PARP, in 3 representative cell lines (human- U118 and KT21; canine SDT3g) following exposure to PAC-1 for 24 h at 0, 6.25, 12.5, 25, and 50 μ M (gradient). Variable processing of PARP represented by PARP degradation only (SDT3g), generation of cleaved PARP only (KT21), or combination (U118). Black arrowhead (PARP); red arrowhead (cleaved PARP).

TABLE 2 | Cell lines used in this study.

Cell Line	Species	Cell line type	72h PAC-1 IC50 (μ M)
U87-MG	Human	Glioma	15.2 $\pm$ 2.8
U118-MG	Human	Glioma	19.9 $\pm$ 7.7
IOMM	Human	Meningioma	4.25 $\pm$ 0.7
KT21	Human	Meningioma	6.5 $\pm$ 0.5
GO6A	Canine	Glioma	14.9 $\pm$ 6.1
J3TBg	Canine	Glioma	3.3 $\pm$ 0.1
SDT3g	Canine	Glioma	10.7 $\pm$ 3.7

mm³ (range 1.3–141.2 mm³; **Figure 5D**); however, the relative tumor-fold increases failed to reach statistical significance.

Procaspase-3 IHC Expression Correlates With Tumor Histologic Grade and Survival

Across CNS malignant histologies in humans, PC-3 expression was strongest in high-grade astrocytomas. To investigate whether PC-3 IHC grading could correlate with survival in human patients diagnosed with astrocytomas, a subset of 157 samples from Johns Hopkins University with clinical outcome-linked data available were evaluated. Survival correlated, as expected, with WHO histologic grade (**Figure 6A**). Importantly, PC-3 expressions, either graded or dichotomously categorized (**Figure 6B** or **Figure 6C**, respectively), correlated with decreased patient survival (**Table 3**). Log rank tests were statistically significant in all cases ($p < 0.001$).

DISCUSSION

Malignant gliomas in humans remain clinically challenging to treat given the invasive nature of tumor cells into normal surrounding brain parenchyma, which precludes the feasibility of complete surgical resection in most affected patients. As such, conventional standard-of-care therapy for malignant gliomas in humans is trimodal in nature, inclusive of maximal surgical resection without the creation of unacceptable neurologic deficits, definitive radiation therapy, and adjuvant chemotherapy (2). Despite a modest therapeutic advance following the introduction of temozolomide therapy in 2005, the median survival time in humans with GBM receiving trimodal therapy is disappointingly short, and has remained static at only 14 months (40). To potentially improve outcomes for patients diagnosed with GBM, new therapies which are capable of penetrating into the central nervous system compartment and that preferentially induce apoptosis of cancer cells must be identified.

Resistance to normal cellular apoptosis is a hallmark of tumorigenesis, and there are numerous intracellular proteins—including death receptors, mitochondrial pore proteins, and TP53, that can be upregulated or downregulated, leading to inhibition of normal cellular apoptosis in cancer cells (41). Many efforts have been made to create therapeutics that target dysregulated molecular targets in the apoptotic cascade, yet none have clinically improved outcomes in people with aggressive astrocytomas, such as GBM. Regardless of where upstream cellular mutations occur, both extrinsic and intrinsic arms of the apoptotic cascade converge on the activation of procaspase-3

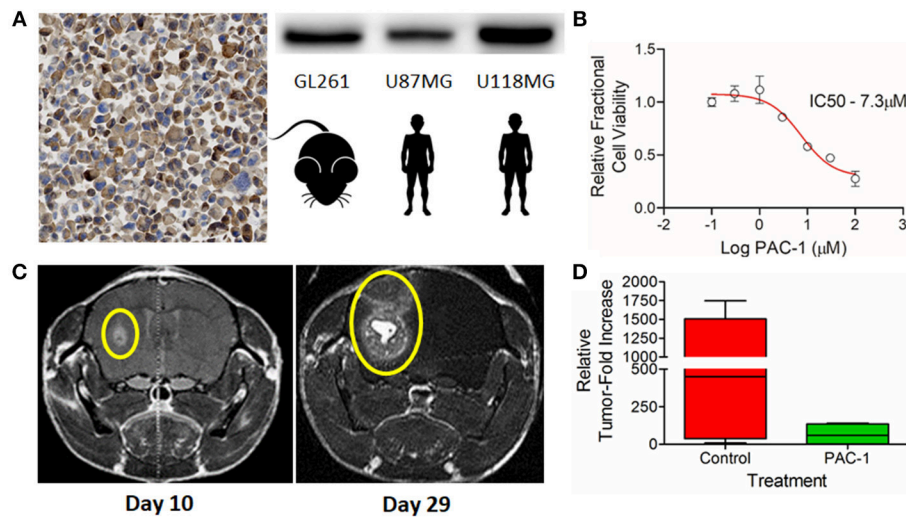


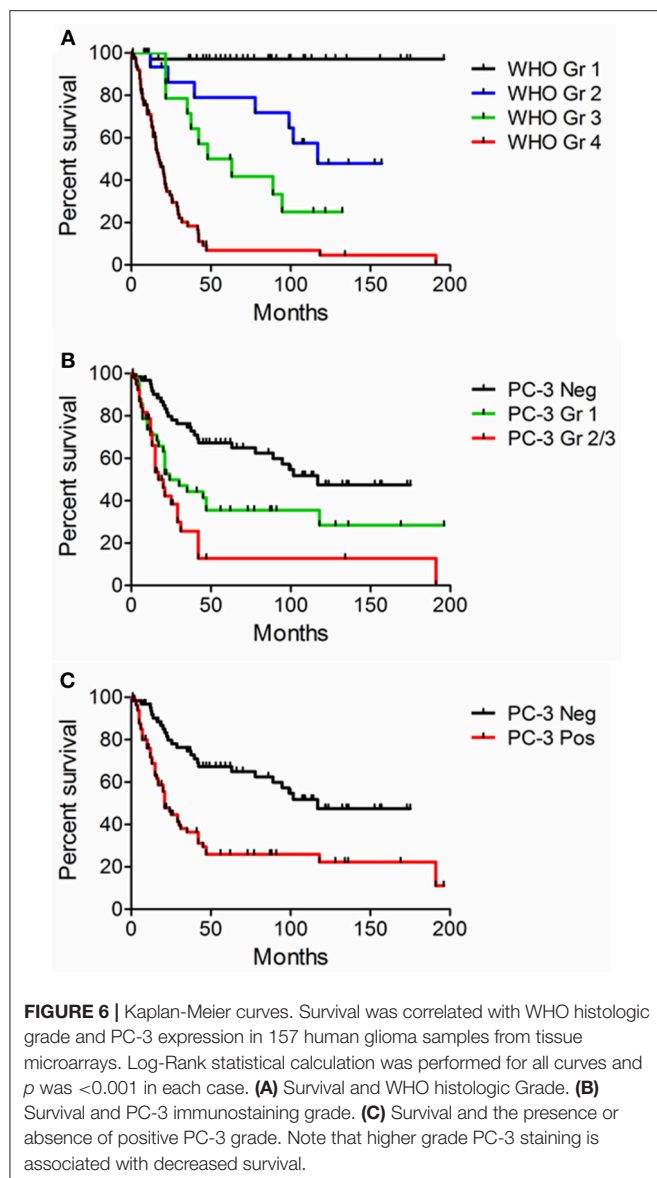
FIGURE 5 | (A) Immunohistochemical and western blot detection of PC-3 in the murine glioma cell line, GL261; positive human controls U87MG and U118MG included as comparative references. **(B)** GL261 *in vitro* dose-dependent sensitivity to PAC-1 following 72 h of exposure. **(C)** Reliable induction of orthotopic tumors 10 days following the stereotactic-guided intracranial injection of 50,000 GL261 neurospheres (Left) with subsequent rapid growth over a period of 19 days (Right); gadolinium contrast enhancing tumors identified with yellow ellipses. **(D)** Variable attenuation in GL261 orthotopic growth in mice receiving oral PAC-1 (100 mg/kg) twice daily for 10 treatments compared to sham vehicle treated mice; difference not statistically significant.

(PC-3) to caspase-3, the key executioner protease within the cell. Therefore, as a BBB penetrant PC-3 activator, PAC-1 is uniquely positioned as a therapeutic that can circumvent the wide range of upstream apoptotic evasion strategies adopted by cancer cells; and molecularly, it functions via direct chelation of labile cellular zinc with consequent caspase-3 activation and induction of apoptotic cell death (35, 42). While PAC-1 possesses mechanistic advantage for directly activating caspase-3, the existence and dysregulation of IAP family of proteins, such as XIAP residing downstream of executioner caspases that can directly neutralize activated executioner caspases via proteasome degradation (43), could attenuate the selective anticancer apoptotic properties of PAC-1.

While conceptually attractive to induce programmed cell death through direct PC-3 activation, a concern of using a potent pro-apoptotic compound to treat brain cancer is the potential for off-target effects, resulting in cell death in normal brain tissue. Indeed, PC-3 is ubiquitously expressed in the body, and within the developing nervous system, there is an obligate need for programmed cell death to remove unnecessary neurons and for pruning of neuronal synapses (44, 45). Additionally, caspase-3 activity in C57BL/6 mice has been shown to be important in cognition and behavior, particularly inhibitory control, and is a key player in synaptic homeostasis (46). In the current study, PC-3 was identified in normal brain structures in both humans and dogs, albeit at much lower expression levels in comparison to malignantly transformed tissues. By immunofluorescence, PC-3 was identified in synaptic-like structures, and it is plausible that this observed localization may indicate participation in synaptic homeostasis, as identified in mice, in higher mammalian species as well. Despite low expressions of PC-3 identified in normal brain tissues, the observation for histopathologic off-target effects

in brain tissues have not been observed in preclinical toxicity studies with healthy rodents receiving orally administered PAC-1 at clinically relevant dosages. More importantly and translationally relevant, PAC-1 has been well-tolerated clinically and neurologically in dogs with spontaneous cancers including gliomas, as well as in humans diagnosed with diverse tumor histologies (34, 47, 48).

In this study, the potential for PAC-1-mediated PC-3 activation is very pertinent to human oncology, as PAC-1 induces cell death in cancer cells in proportion to the resting PC-3 concentration within the cell (38), and our findings support PC-3's overexpression in the majority of both human and canine brain tumors relative to normal tissues. Given the observed differential in PC-3 expressions, it remains plausible that a therapeutic window exists whereby PAC-1 would preferentially induce apoptosis in malignantly transformed cells, yet spare surrounding brain tissues with substantively lower PC-3 expressions. To guide the identification of a therapeutic PAC-1 concentration range for brain cancer therapy, we showed that low micromolar concentrations of PAC-1 lead to cell death across a panel of immortalized cell lines from both species. However, sensitivity to PAC-1 *in vitro* was not identified to be directly proportional to resting PC-3 alone or in the context of XIAP, a known IAP involved in glioma apoptosis resistance (33, 49, 50). Given the multitude of cellular parameters that might influence apoptosis susceptibility and resistance, these findings are not completely unexpected, but rather underscore the complexity of opposing cellular death promoting- and resisting- pathways. Nonetheless and importantly, these *in vitro* concentrations of PAC-1 that reliably induced cancer cell apoptosis are readily achievable in rodents, dogs, and human beings *in vivo* (48, 51). While our *in vitro* data support the role of PAC-1 as a single agent,



the greatest clinical benefit to brain cancer patients will likely be achieved through the inclusion of PAC-1 as an adjuvant therapy, a supposition supported by PAC-1's demonstrated ability to be safely combined and synergize with diverse agents, including radiation therapy, other apoptosis-activating agents, and temozolomide (34, 47, 52).

In addition to PC-3 serving as an attractive therapeutic target, our findings provide preliminary support for the prognostic significance of PC-3 for some brain tumor pathologies. In 157 human astrocytoma samples with outcome linked clinical information, increased expression of PC-3 correlated with increasing histologic grade and decreasing survival time. These observed correlations might have two profound clinical implications. First, because some astrocytomas do not express PC-3 (67 out of 157 samples), there would be molecular justification to stratify cohorts of patients to receive or not receive PAC-1 based upon PC-3 expressions, similar to what is already

TABLE 3 | WHO histologic grade, survival, and PC-3 grade.

WHO histologic grade	Grade 1	Grade 2	Grade 3	Grade 4
# Cases	38	19	14	86
% Censored	97.4%	63.2%	28.6%	26.7%
MST (Months)	N/A	58.7	37.5	15.3
PC-3 grade	Negative	Grade 1	Grade 2 or 3	
# Cases	67	45	45	
% Censored	59.7%	42.2%	37.8%	
MST (Months)	24	17	15.3	
PC-3 staining	Negative	Positive		
# Cases	54	66		
% Censored	51.8%	18.2%		
MST (Months)	25.4	15.3		

conventionally practiced for the institution of temozolomide based upon tumoral MGMT hypermethylation status. Second, given the inverse correlation between PC-3 and survival time, these findings imply that PC-3 activating strategies might provide the greatest potential benefit in patients with the gravest prognoses, and adjuvant PAC-1 therapies might maximally extend survival times when instituted early in the planned treatment course for affected patients.

Pet dogs with spontaneous tumors provide a unique comparative opportunity to model human disease, including certain types of brain cancer. Dogs often develop spontaneous brain tumors that are histologically indistinguishable from those seen in humans, and can afford the scientific community with a unique model system to study and characterize novel treatment strategies or devices for improving brain cancer management (10, 16, 25, 34). While significant justification exists for the inclusion of comparative oncology for expediting drug development efforts, there are some recognized limitations in using canines as a model of human brain cancer. For example, in humans but not in dogs, meningioma COX-2 expression correlates with proliferative index and tumor grade (53, 54), exonic p53 mutations are more common in human than in canine astrocytomas (55), and there is no correlation between meningioma grade and NF2 expression in canine tumors as is seen in humans (56). As such, leveraging the unique aspects of comparative oncology should be tailored to the most appropriate disease pathologies, and not considered a "catch all" parallel modeling system.

Given the aggregate data generated in our study, PC-3 appears to be a valid and druggable target for specific brain tumor histologies, particularly high grade astrocytomas; and small molecule activators of PC-3, such as PAC-1, should be further explored for their clinical utility for improving the management of brain tumors overexpressing PC-3. Several chemical and pharmacologic properties of PAC-1 are attractive for the management of brain cancer including its oral administrative route, achievement of predicted therapeutic concentrations which are safe in humans and dogs (34, 47, 48, 51), and ability to traverse the BBB (36). Collectively, these data and properties of PAC-1 have led to the conductance of a Phase

1b trial of PAC-1 plus temozolomide in refractory glioblastoma and anaplastic astrocytoma patients (NCT02355525). Although additional study and *in vivo* modeling are needed, these initial data show great promise for PAC-1 as a therapeutic for intracranial neoplasms and for the inclusion of pet dogs with brain cancer as a unique modeling resource for the scientific community.

ETHICS STATEMENT

This study utilized archived human and canine patient samples for histologic assessment, no living patient (human or canine) were used for this study. All human samples were deidentified.

AUTHOR CONTRIBUTIONS

PH and TF: project conception and supervision of work. TF, LS, and SL: experimental design. LS, HP, CE, and PD: sample

preparation. LS, BF-A, HP, and SL: experimental procedures. LS and TF: prepared the manuscript with support from AL.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Human Ovarian Cancer Tumor Formation in Severe Combined Immunodeficient (SCID) Pigs

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Ovarian cancer (OvCa) is the most lethal gynecologic malignancy, with two-thirds of patients having late-stage disease (II-IV) at diagnosis. Improved diagnosis and therapies are needed, yet preclinical animal models for ovarian cancer research have primarily been restricted to rodents, for data on which can fail to translate to the clinic. Thus, there is currently a need for a large animal OvCa model. Therefore, we sought to determine if pigs, being more similar to humans in terms of anatomy and physiology, would be a viable preclinical animal model for OvCa. We injected human OSPC-ARK1 cells, a chemotherapy-resistant primary ovarian serous papillary carcinoma cell line, into the neck muscle and ear tissue of four severe combined immune deficient (SCID) and two non-SCID pigs housed in novel biocontainment facilities to study the ability of human OvCa cells to form tumors in a xenotransplantation model. Tumors developed in ear tissue of three SCID pigs, while two SCID pigs developed tumors in neck tissue; no tumors were detected in non-SCID control pigs. All tumor masses were confirmed microscopically as ovarian carcinomas. The carcinomas in SCID pigs were morphologically similar to the original ovarian carcinoma and had the same immunohistochemical phenotype based on expression of Claudin 3, Claudin 4, Cytokeratin 7, p16, and EMA. Confirmation that OSPC-ARK1 cells form carcinomas in SCID pigs substantiates further development of orthotopic models of OvCa in pigs.

Keywords: ovarian cancer, severe combined immunodeficient, swine, preclinical animal model, Claudin

INTRODUCTION

Ovarian cancer (OvCa) is the most lethal among gynecologic malignancies, taking an estimated 14,000 lives in the United States in 2018 (1). OvCa often goes undetected until late stages due to non-specificity of its early symptoms, hence 2/3 of patients have late-stage disease (stage III-IV) at diagnosis. The current standard of care is debulking surgery to remove tumor masses followed by first-line platinum and Taxol chemotherapy (2). Debulking is critical to successful chemotherapy, and so prior identification of tumor masses by diagnostic imaging often plays a key role in pre-surgical

planning. X-ray computer tomography (CT) (3) is the most widely used imaging modality for evaluating peritoneal spread in OvCa for presurgical planning, yet there are well acknowledged “blind-spots” where tumor spread simply cannot be seen as the contrast between normal tissue and tumors is insufficient to discriminate one tissue type from another. Thus, tumors can be missed, leading to incomplete tumor resection. Methods to enhance tumor detection are being developed for a variety of imaging modalities, including magnetic resonance imaging (MRI) and positron emission tomography (PET), making use of various targeting mechanisms to specifically target ovarian cancers. For example, small peptides and molecules including OTL38 (4), GE11 (5), and CPE (6–9), which bind to folate receptor, EGFR, and claudins, respectively, have been successfully tested in preclinical mouse trials. Clinical trials for use of OTL38 are already beginning to recruit patients with folate receptor positive ovarian cancer for the use of this fluorescent molecule during cytoreduction or debulking surgeries (10).

Current research to improve imaging technologies and methodologies uses either human volunteers or rodent models. Imaging research involving human OvCa patients is challenging for a variety of reasons. For imaging modalities which involve significant radiation, such as CT, extensive research cannot be performed due to radiation dose. Second, it is challenging to perform serial imaging research on OvCa patients due to ongoing treatment regimens and patient morbidity. Such serial scans could be useful in developing predictive imaging capability derived from a multiparametric data set (11). Development of imaging strategies on rodents poorly informs how one approaches the clinical scenario. This is due to the size of the animal and the tumors, as well as the equipment that are used in animal imaging experiments. Small tumors exhibit different perfusion (12) and water diffusion (13) from large tumors. Small animals have different metabolism than large animals and exhibit vastly different pharmacology of administered drugs (14), while humans and pigs have similar liver content of cytochrome proteins (15, 16). All of these characteristics, and many more, influence imaging results.

It is not always practical to use human OvCa patients for developing or validating new imaging techniques, and rodents are inadequate for developing clinically relevant imaging protocols. Recently there is a new hybrid field of molecular imaging and surgery called optical surgical navigation (17). This field couples fluorescence imaging with surgery to enhance surgical removal of tumors by way of fluorescent marker uptake. The translational value of new fluorescent tracers, either targeted or untargeted, can only be meaningfully evaluated in the context of a research subject that has appropriate size and physiology to OvCa patients. A pig model of OvCa could fill this crucial gap.

Human cancer xenotransplantation studies have not been possible in pigs until the recent identification (18) or creation (19–22) of severe combined immunodeficient (SCID) pigs (23). SCID pigs have previously been reported to accept grafts of human melanoma (A375SM) and pancreatic carcinoma (PANC-1) cancer cell lines (24), as well as human induced pluripotent stem cells (25). In addition to xenotransplantation methods of studying cancer, genetic models of porcine cancer have also

been developed. Inducible and germline mutations of TP53^{R167H} (26, 27) and KRAS^{G12D} (28, 29) have been introduced into pigs, which are useful in studying lymphomas, osteogenic tumors, renal tumors, and others. These neoplasms were detected with computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound imaging systems (27), exemplifying the pig's utility as an imaging animal model system.

To initiate the development of a large animal model of ovarian cancer, we tested whether human ovarian cancer cells could survive and develop ectopic tumors in SCID pigs. OSPC-ARK1 cells, derived from an ovarian serous papillary carcinoma (OSPC), were injected into male and female SCID pigs and were monitored for tumor development for this first stage screen. We demonstrate that OSPC-ARK1 derived carcinomas developed in three of four SCID pigs tested. Additionally, we verified an immunophenotype comparable to human patient OSPC samples based on the expression of Claudin 3, Claudin 4, Cytokeratin 7, p16, and EMA in SCID pig carcinomas. In summary, we demonstrate that SCID pigs can successfully develop OSPC-ARK1 carcinomas, which warrants further development of an orthotopic SCID pig model of ovarian cancer.

MATERIALS AND METHODS

Generation and Care of Piglets

Wildtype and ART^{-/-} piglets were generated as described (18) and were housed in positive pressure biocontainment bubble facilities (30). All animal protocols and procedures were approved by Iowa State University's Institutional Animal Care and Use Committee (IACUC).

Human Tissue Collection

Informed consent was obtained from human subjects and was approved by the Yale Institutional Review Board. The OSPC-ARK1 primary ovarian cell line used in this study was established from samples collected at the time of tumor recurrence from a patient harboring stage IV ovarian serous papillary carcinoma.

Cell Preparation and Injections

SCID Pigs

OSPC-ARK1 cells were grown in complete RPMI media (10% FBS, 50 µg/mL gentamycin, 10 mM HEPES) until 80–100% confluent. Cells were trypsinized and washed five times in sterile phosphate buffered saline (PBS). Cells were then counted with a hemocytometer and were brought to a concentration of 50×10^6 cells/mL.

Animals were anesthetized with isoflurane. A total of six pigs were used in two independent experiments. Injection sites were marked and all animals were injected subcutaneously in the right and left ear and intramuscularly into the right and left side of the neck. In the first trial, four 43 day old pigs (S1, S2, NS1, and NS2) were injected with 5 million cells in a 100 µL PBS cell suspension. In the second trial, two 18 day old SCID pigs (S3 and S4) were injected with the same number and volume of cells in the same locations. The SCIDs in trial 1 were female, while the SCIDs in trial 2 were male. **Table 1** shows an overview of piglet ID,

sex, trial, genotype, age at trial end, and locations of carcinoma formation.

SCID Mice

C.B-17/SCID female mice 5–7 weeks old were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and housed in a pathogen-free environment. They were given basal diet and water *ad libitum*. All animal protocols and procedures were approved by Yale University's IACUC. OPSC-ARK1, a chemotherapy-resistant primary ovarian serous papillary carcinoma cell line, was used to develop a xenograft models. OPSC-ARK1 cancer cell line was injected IP at a dose of 7 million cells.

Tissue Collection and Processing

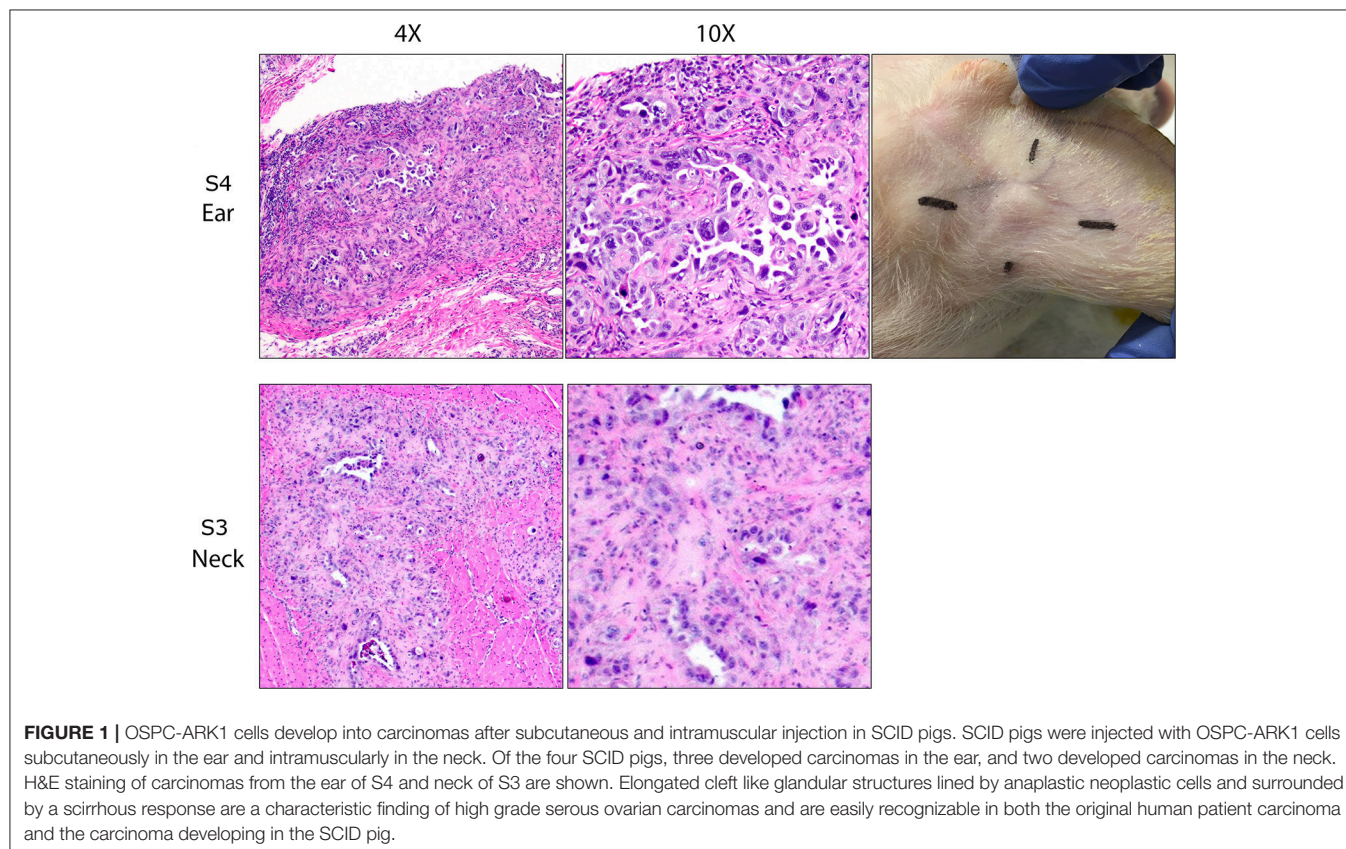
Tissues were collected at the marked injection sites and fixed in 10% neutral buffered formalin for 24 h, at which point they were transferred to 70% ethanol. Following routine processing, tissues were embedded into paraffin and serial sections were cut at 5 μ m thickness. Slides were either stained with hematoxylin and eosin or used for immunohistochemical labeling. In addition, OPSC-ARK1 cells were harvested and fixed in 10% neutral buffered formalin for 24 h and transferred to 60% alcohol. Following centrifugation, cell pellets were harvested and suspended in liquid histogel (Thermo Fisher Scientific, Ann Arbor, MI, USA). After the histogel had solidified, samples were transferred to 60% alcohol followed by routine tissue processing and embedding into paraffin. Serial sections were cut at 5 μ m thickness and processed in parallel to the tissue samples.

TABLE 1 | SCID and non-SCID pig descriptions and tumor growth locations.

Pig ID	Sex	Trial #	Days on trial	Age at end of trial	R Ear	L Ear	R Neck	L Neck
S1	F	1	13	56 d	+	–	+	–
S2	F	1	30	73 d	–	–	–	–
S3	M	2	11	29 d	+	+	+	+
S4	M	2	7	25 d	+	–	–	–
NS1	M	1	30	73 d	–	–	–	–
NS2	M	1	30	73 d	–	–	–	–

Immunohistochemistry

Serial sections of the pig tumors and the cell line were routinely labeled with immunohistochemistry for Claudin 3, Claudin 4 (both Thermo Fisher Scientific, Ann Arbor, MI, USA), Cytokeratin 7 (Agilent, Santa Clara, CA, USA), p16 (BD Biosciences, Franklin Lakes, NJ, USA), and EMA (LifeSpan BioSciences, Seattle, WA, USA). In addition, OPSC-ARK1 xenotransplant tumors from mice and sections of the original biopsy of this neoplasm that had been processed in a similar manner as the pig tissues were run as controls.



Immunohistochemistry was performed on the Dako link 48 Automated Staining System (Agilent Technologies, Santa Clara, CA, USA) using the peroxidase conjugated EnVision Polymer Detection System (Agilent Technologies, Santa Clara, CA, USA) for all antibodies. Briefly, endogenous peroxidases were neutralized with 3% hydrogen peroxide for 5 min. Antigen retrieval was achieved by incubating slides in either low pH (Claudin 3, Claudin 4 and EMA) or a high pH (EMA) retrieval solution for 20 min on the Dako PT link (Agilent Technologies, Santa Clara, CA, USA) or through 20 min of protein digestion with proteinase K (Cytokeratin 7). Non-specific immunoglobulin binding was blocked by incubation of slides for 10 min with a protein-blocking agent (Agilent Technologies, Santa Clara, CA, USA). Using the Dako autostainer, slides were incubated for 30 min with a rabbit polyclonal anti-human Claudin 3 antibody (#34-1700), a mouse monoclonal anti-human Claudin 4 antibody (clone 3E2C1), a mouse monoclonal anti-human Cytokeratin 7 antibody (clone OV-TL 12/30), a rabbit polyclonal anti-human EMA antibody (#LS-C30532) and a mouse monoclonal anti-human p16 antibody (clone G175-405) at dilutions of 1:100, 1:250, 1:75, 1:500, and 1:100, respectively. The immunoreactions were visualized with 3,3-diaminobenzidine substrate (Dako, Carpinteria, CA). Sections were counterstained with Mayer's haematoxylin.

RESULTS

OSPC-ARK Cells Injected Into SCID Pigs Develop Carcinomas

To assess if human ovarian carcinomas could develop in SCID pigs, a total of four SCID (S1, S2, S3, and S4) and two non-SCID (NS1 and NS2) pigs were injected with OSPC-ARK1 cells. Due to limited female SCID availability, we injected two female and two male SCID pigs in this initial study to answer our question of if OSPC-ARK1 cells were capable of developing tumors ectopically in immunocompromised pigs. Four sites were injected in each pig; one subcutaneous on each ear, and one intramuscular on each side of the neck. We decided to inject in these superficial sites such that we could easily and noninvasively monitor tumor growth over time. Evidence of growth in these sites would warrant injection into a more physiologically relevant area, such as the peritoneum or ovary of female SCID pigs.

Palpable tumors were observed on the ears of three SCID pigs prior to euthanasia. S1 and S2 were euthanized at 13 and 30 days after neoplastic cell inoculation and no grossly visible tumors were observed on the neck sites of injection. S3 was euthanized 11 days after injection and tumors were observed on the left and right ears. S4 was euthanized 7 days after injection, at which point a tumor was detected on the right ear (**Figure 1**). Wild-type animals, NS1 and NS2, did not have visible tumors at 30 days post injection. **Table 1** shows an overview of locations of carcinoma development in the six pigs.

At euthanasia, samples from each injection site were collected and fixed, and H&E staining and analysis was used to determine if tumor architecture was present. Of the four SCID pigs injected, ovarian carcinomas were present in three animals in at least one

injection site. S1 and S3 (**Figure 1**) developed carcinomas in the neck. S1, S3, and S4 all developed carcinomas within the ear tissue. S3 had carcinoma present in all four injection locations. In all cases neoplastic cells incited and were surrounded by an extensive scirrhous response. Most commonly, neoplastic cells formed small nests, solid cords or elongated cleft like glandular structures that were lined by anaplastic neoplastic cells. There was marked anisocytosis and anisokaryosis and the degree of cellular pleomorphism and the remarkable scirrhous response are characteristic findings of high grade serous ovarian carcinomas. In summary, we were able to demonstrate that OSPC-ARK1 cells were able to successfully form ovarian carcinomas in SCID pigs.

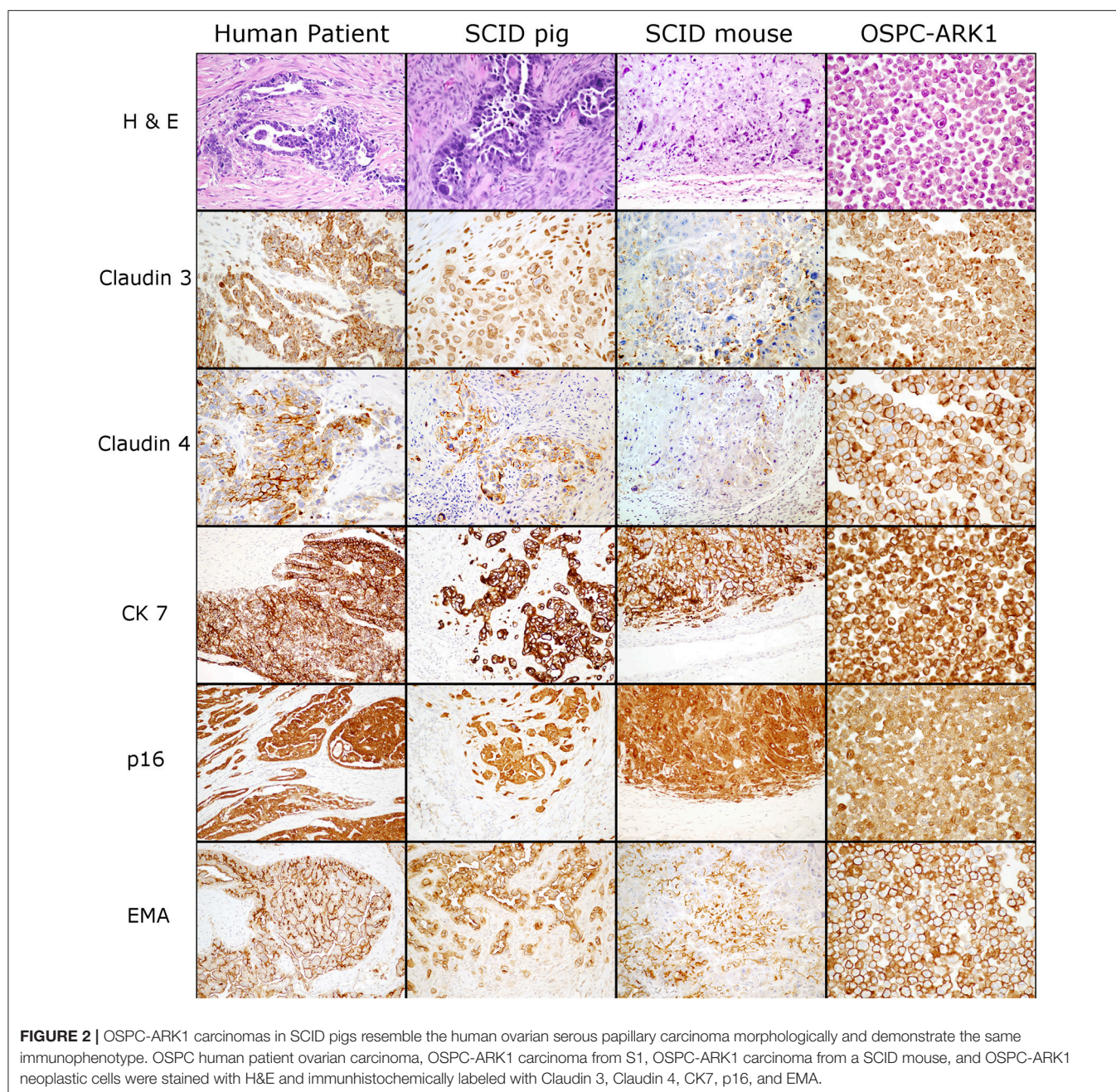
OSPC-ARK1 Carcinomas in SCID Pigs Maintain Expression of Common Ovarian Carcinoma Diagnostic Markers

We next wanted to determine if ovarian carcinoma protein marker expression were retained in the pig xenotransplants. OSPC-ARK1 cells, the original human carcinoma (neoplasm from which the OSPC-ARK1 cell line was derived), and OSPC-ARK1 derived carcinomas in SCID pigs and SCID mice were subjected to immunohistochemical analysis (**Figure 2**). S1 (female) is shown in **Figure 2**. We assessed the expression of p16, epithelial membrane antigen (EMA), cytokeratin 7 (CK7), which have previously been used in diagnostic panels (31); we also assessed expression of Claudin 3 and 4 in all samples. Expression of CK7, p16, and EMA were all highly similar in tissue samples from all three species, as well as in the pellets generated from the OSPC-ARK1 cell line. Importantly, Claudin 3 and 4 expression in SCID pig carcinomas was also highly similar to the observed expression pattern in the original human carcinoma. In summary, OSPC-ARK1 carcinomas in SCID pigs have the same immunophenotype as the original ovarian carcinoma from a human patient.

DISCUSSION

We have described the successful development of human OSPC-ARK1 carcinomas in SCID pigs. Injected sites were verified histopathologically as true carcinomas. We additionally showed that tumors in SCID pigs phenotypically resembled human ovarian carcinomas through assessing the expression of OvCa protein markers CK7, p16, and EMA (31). Furthermore, we showed that tumors in SCID pigs retained expression of Claudin 3 and Claudin 4, which we have previously used as an imaging and therapeutic target in mouse models (6–8).

The ability of human ovarian tumors to grow in SCID pigs warrants further development of an orthotopic model of this cancer. The capability to study a human tumor in a non-rodent species is critically important as it would allow researchers and medical practitioners to utilize imaging modalities that are used in clinical settings. Additionally, there are many cases where progression from early-stage to late-stage occurs rapidly and can often happen within the span of a few months. We have raised SCID pigs for up to 6 months (unpublished results) in



biocontainment facilities (30), which would allow long term trials that are required for studying spread and metastasis to be performed. The SCID pigs used in this study have natural mutations in *ARTEMIS* (18), which is a critical component of the VDJ recombination pathway required for TCR and BCR development, and thus have a $T^- B^- NK^+$ cellular phenotype. NK cells are functional in our SCID model in *in vitro* assays (32), which could have anti-tumor activity on human cancer cells as evidenced by the absence of tumor development in S2. Thus, use of a $T^- B^- NK^-$ SCID pig may better facilitate human ovarian tumor growth.

Pigs and humans share more similar reproductive tract sizes and structures than mice. In this initial trial, tumor growth from the OSPC-ARK1 cell line was not dependent on the sex of the animal. However, as we develop this model further, we would inject OSPC-ARK1 cells into the peritoneum or ovarian bursa in female SCID pigs. Such orthotopic tumor sites would allow for new imaging research to be initiated in SCID pigs. Studies involving surgical practices cannot be efficiently performed in mice because tumors are too small. Moving forward, it will be an important step to test human specific imaging targets (CPE, folate, GE11) and

systems (PET, MRI) in SCID pigs xenografted with human tumors.

We have previously utilized the CPE peptide to either label tumors with a fluorescent marker (8) or deliver a suicide gene (9) to the site of ovarian tumors mice. Inoculation of OSPC-ARK1 cells into SCID pigs would allow for methods, dosages, efficacy, and safety of the CPE peptide to be established. Additionally, injection of fluorescently labeled CPE would allow for surgical practices to be performed for use of this peptide in marking smaller tumors that are difficult to detect by commonly used imaging practices. The pigs would be of comparable size to humans, so dosages of the peptide would be relevant as well. In all, confirming that human ovarian carcinomas can successfully develop in SCID pigs provides a basis for further development of an orthotopic OvCa model in pigs.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Yale University & Institutional Review Board with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Yale University & Institutional Review Board. This study was carried out in accordance with the recommendations of ARRIVE and PREPARE guidelines recommended by the Institutional Animal Care and Use Committee. These protocols were approved by the Iowa State University

and Yale University & Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

AB was involved in SCID pig cell injections, compiling data, and writing manuscript. MK performed histological and immunohistochemical analyses and provided histological and immunohistochemical descriptions. MA and JaR were involved in OSPC-ARK1 cell preparation. EC, AS, SB, and BB-F were involved with SCID mouse cell injections and human sample collection. SC was involved in SCID pig care and maintenance throughout the trials. JoR was involved in experimental design. CT injected cells into pigs and performed dissections. CT and ES designed experiment and reviewed data. All authors were involved in editing the manuscript.

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Ovine Pulmonary Adenocarcinoma: A Unique Model to Improve Lung Cancer Research

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Lung cancer represents a major worldwide health concern; although advances in patient management have improved outcomes for some patients, overall 5-year survival rates are only around 15%. *In vitro* studies and mouse models are commonly used to study lung cancer and their use has increased the molecular understanding of the disease. Unfortunately, mouse models are poor predictors of clinical outcome and seldom mimic advanced stages of the human disease. Animal models that more accurately reflect human disease are required for progress to be made in improving treatment outcomes and prognosis. Similarities in pulmonary anatomy and physiology potentially make sheep better models for studying human lung function and disease. Ovine pulmonary adenocarcinoma (OPA) is a naturally occurring lung cancer that is caused by the jaagsiekte sheep retrovirus. The disease is endemic in many countries throughout the world and has several features in common with human lung adenocarcinomas, including histological classification and activation of common cellular signaling pathways. Here we discuss the *in vivo* and *in vitro* OPA models that are currently available and describe the advantages of using pre-clinical naturally occurring OPA cases as a translational animal model for human lung adenocarcinoma. The challenges and options for obtaining these OPA cases for research purposes, along with their use in developing novel techniques for the evaluation of chemotherapeutic agents or for monitoring the tumor microenvironment in response to treatment, are also discussed.

Keywords: human lung cancer, jaagsiekte sheep retrovirus, ovine pulmonary adenocarcinoma, sheep lung cancer models, comparative oncology

HUMAN LUNG CANCER

Lung cancer is the most commonly diagnosed cancer in the world, with ~1.8 million new cases and 1.6 million cancer-related deaths recorded each year (1). Lung cancer treatment can be challenging as most patients are diagnosed when the disease is at an advanced stage. Poor response rates to radio- and chemotherapy have meant that overall 5-year survival rates are only 15%. The disease is highly heterogeneous and is divided into several subtypes; their classification

is under periodic review and in 2011 a multidisciplinary classification system was proposed by the European Respiratory Society and International Association for the Study of Lung Cancer (2). Their classification was based on factors such as disease biology, pathogenesis, and histopathology, which rendered terms such as bronchioloalveolar carcinoma (BAC) and its mucinous and non-mucinous forms redundant.

Lung cancer is broadly classified into small-cell lung cancer, originating from bronchial neuroendocrine cells, and non-small cell lung cancer (NSCLC), originating from lung epithelial cells. NSCLC accounts for ~80% of cases and is subdivided into adenocarcinomas, large-cell carcinomas, squamous cell carcinomas, mixed, and undifferentiated tumors (3).

Adenocarcinomas are the most common form of lung cancer, accounting for 40% of cases. Hyperplasia of lung epithelial cells is thought to be the earliest cellular change that occurs in adenocarcinoma tumorigenesis. Termed “atypical adenomatous hyperplasia,” these pre-malignant lesions can accumulate cellular genetic abnormalities causing the cells to become pleomorphic, demonstrating a non-invasive, lepidic growth pattern along alveolar walls (4). Although these growths are known as adenocarcinoma-*in-situ*, complete surgical resection of lesions <30 mm in diameter results in almost 100% of cases gaining 5-year disease-free survival. However, if untreated, these lesions develop into invasive adenocarcinomas. Minimally invasive adenocarcinomas are lesions <30 mm in diameter with an invasive component <5 mm; surgical resection of these lesions is still likely to give an excellent prognosis. The cellular growth pattern (lepidic, acinar, papillary, or solid) is used to classify invasive adenocarcinomas >30 mm in diameter; these invasive forms are the most common clinical and pathological presentation of the disease. Lepidic-predominant adenocarcinoma describes invasive adenocarcinomas that have a predominant lepidic pattern with an invasive component >5 mm (previously termed non-mucinous BAC). In addition, a mucinous form of lepidic adenocarcinoma may also be encountered (previously termed mucinous BAC); this non-invasive, minimally-invasive or invasive disease is often bilateral and multifocal with extensive mucous production. Patients suffering from this subtype present with a cough and extensive mucous production that can lead to death from respiratory failure without any evidence of invasive disease (2).

MOUSE MODELS OF HUMAN LUNG CANCER

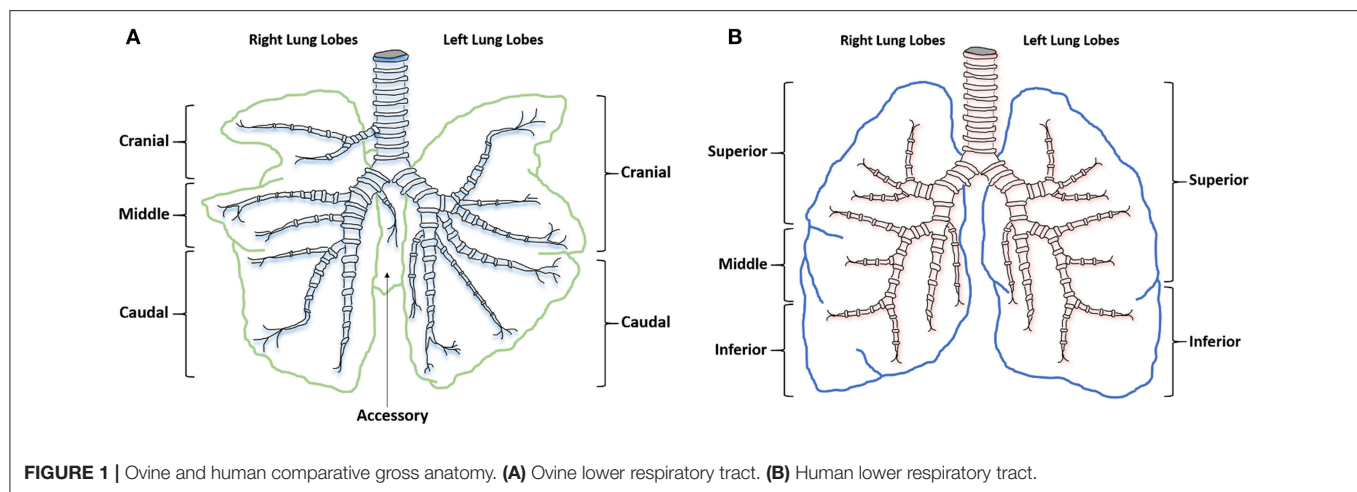
Numerous animal models (primates, dogs, hamsters, mice) have been described for lung cancer research (5, 6). Mice have traditionally been considered the preferred model due to cost-effectiveness and ease of genetic manipulation (7). Many mouse models are now available, including inbred strains exhibiting high rates of spontaneous lung tumors (8–10) (useful for chemoprevention studies), chemical (11)/carcinogen (5)/environmental-induced lung cancer models (12) (allowing the study of tumor initiation and progression) and orthotopic

xenograft models (13–16) (facilitating the analysis of both primary and metastatic tumors). Hundreds of transgenic mouse strains which incorporate the genetic mutations that occur in human lung cancer can now be produced. These mice will produce tumors with greater similarity to human disease and allow the genes that drive lung cancer development and progression to be identified (17). These genetic changes include tumor suppressor gene inactivation (p53, retinoblastoma, and p16), oncogene activation (K-ras), altered growth factor expression (18), loss of heterozygosity, and amplification of specific chromosomal regions (17, 19). The use of bioluminescent or fluorescent reporters in mice is also possible (20, 21). These models allow lineage tracing to be performed and can lead to the identification of individual oncogenes involved in tumorigenesis and can enable the determination of the tumor cell type origin (22).

Despite these advantages, murine models do not accurately represent the advanced stages of lung cancer and are poor predictors of clinical outcome. Each model also has its own specific disadvantages, such as a lack of metastasis in genetic and chemically induced models and the inability to examine immune response in tumor development/progression in xenograft models that require the use of immunodeficient mice (7). The perceived advantages of having multiple models can also be seen as a limitation, as no one single model can be used to examine all stages of the disease.

COMPARATIVE HUMAN AND SHEEP PULMONARY ANATOMY AND PHYSIOLOGY

Similarities between human and sheep pulmonary anatomy and physiology has led to sheep being identified as an excellent model for investigating human lung function and disease. Human lung anatomy consists of the left lung divided into superior and inferior lobes and the right into superior, middle and inferior lobes. Sheep anatomy is similar with the left lung divided into cranial and caudal lobes and the right into cranial, middle, caudal, and accessory lobes. In sheep each lobe is separated by tissue septa, which limits lobular connectivity (23) (**Figure 1**). Although in sheep the right cranial lobe bronchus arises directly from the trachea before the tracheal bifurcation (24), with respiratory bronchioles that are poorly developed (23) the remaining tracheobronchial tree is similar in both species, showing an irregular dichotomous branching pattern. The distribution of differentiated respiratory epithelial cells (25), mast cells (26), and airway smooth muscle (27) is also comparable between the species. Although human lungs have fewer intravascular macrophages compared with the large number seen in sheep lungs (28), increased numbers can occur after an endotoxic insult. Lung development is also similar between the species; lamb lungs show significant similarities to human infant lungs, including prenatal alveologenesis, airway branching patterns, bronchiolar club cell number, type II alveolar epithelial



(pneumocytes) development, and the presence of airway submucosal glands (29).

Similarities in lung size allow sheep models to be used in ways not available in mouse models; techniques including drug administration, advanced imaging (30), ultrasound (31), endoscopy, and surgical procedures can be used in sheep as they would in humans (32). With the correct animal handling facilities, where appropriate, procedures can be performed in conscious or minimally sedated animals, rather than using general anesthesia. Repeated blood sampling and tissue collection is easier in sheep and their longevity allows chronic conditions to be modeled, while also enabling the evaluation of long-term treatments. These factors make sheep excellent models for human respiratory conditions (24) such as asthma (33), cystic fibrosis, chronic obstructive respiratory disease (34), respiratory syncytial virus infection (35), and now cancer (36).

OVINE PULMONARY ADENOCARCINOMA

Ovine pulmonary adenocarcinoma (OPA) is a neoplastic lung disease caused by the jaagsiekte sheep retrovirus (JSRV) (37–40). This betaretrovirus is the only known virus capable of inducing the formation of naturally occurring lung adenocarcinomas. Since the disease was first described in South Africa in the nineteenth century (41), JSRV infection has been identified in numerous sheep breeds and small ruminants throughout the world, the virus however has never been shown able to infect humans (42, 43). Although natural JSRV infection can occur in goats this rarely results in tumor formation and experimental infection of goat kids induces tumors with a different macroscopic and histological appearance to those seen in lambs (44). OPA is endemic in the UK and represents a major economic and animal welfare concern (39, 45). Within-flock disease incidence levels can be as high as 30%, although levels of 2–5% are more common (46). Mortality rates of 50% can be seen following initial disease identification within a flock (47); however, as the disease becomes endemic rates reduce to 1–5% (41, 48). Disease transmission occurs predominantly

through the aerosol route (41, 47, 49), meaning close contact with infected sheep is a significant risk factor. The virus has been detected in the milk and colostrum of infected ewes, which poses a potential source of infection for new born lambs (50).

JSRV BIOLOGY

JSRV particles contain two copies of single-stranded positive sense RNA. Its genome of ~7,460 nucleotides contains four genes encoding viral proteins (39). These four genes are: *gag* (encoding the matrix, capsid, and nucleocapsid proteins); *pro* (encoding aspartic protease); *pol* (encoding reverse transcriptase and integrase enzymes); and *env* (encoding surface and transmembrane envelope glycoproteins) (51, 52). An additional open reading frame, known as *orfX*, which overlaps with the *pol* gene, has also been identified; however, it is not required for *in vitro* cellular transformation (53) or *in vivo* oncogenesis (54–56). Interestingly, JSRV-induced neoplastic transformation is mediated by the viral Env glycoprotein, although the mechanisms underlying this process are not completely understood. The transforming activity of Env was first shown *in vitro* using rodent fibroblasts (53, 57), with subsequent *in vivo* experiments showing that the administration of viral vectors expressing Env to the lungs of mice (56) and sheep (55) results in adenocarcinoma formation. Env localization at the plasma membrane may enable it to interact with other molecules such as protein kinases (58), leading to the activation of downstream pathways that promote cellular proliferation and survival. The Ras-MEK-ERK (59, 60) and PI3K-AKT-mTOR (59, 61, 62) pathways are commonly activated in OPA tumors; others may include EGFR, RON-HYAL2 and heat shock proteins (63). Following pathway activation, it is likely that further mutations are required for tumors to develop, such as telomerase activation (62), the activation of other cellular oncogenes or the inactivation of tumor-suppressor genes. For a detailed description of JSRV structure and replication cycle see the recent review by Youssef et al. (36).

ENDOGENOUS RETROVIRUS AND IMMUNE RESPONSES

Endogenous retroviruses are viruses that have become integrated into host germ-line DNA and are passed through the generations. The sheep genome contains numerous endogenous JSRV (enJSRV) related proviruses with over 90% sequence similarity to exogenous JSRV (exJSRV) (64, 65). These enJSRV proviruses are not oncogenic (they lack the oncogenic Env c-terminal domain present in exJSRV) (37, 51, 66, 67), but are transcriptionally active, with studies showing viral RNA and protein expression in the female reproductive tract and in fetal tissues (67, 68). The expression of these viral proteins may help protect the host from exJSRV infection, either by receptor competition or through the prevention of exJSRV viral particle transport and cellular exit (68, 69).

JSRV infection lacks a specific cellular or humoral immune response to viral proteins. Although neutralizing antibodies specific for JSRV have been found in a minority of infected animals (44, 70), the lack of a consistent adaptive response is likely due to sheep being immunologically tolerant of JSRV antigens as a result of the expression of enJSRV proteins in the fetal thymus during T lymphocyte development. Tumor cells also downregulate the expression of class I antigens of the major histocompatibility complex, preventing their recognition by CD8⁺ T lymphocytes. The influx of alveolar macrophages following JSRV infection, which produce large amounts of interferon gamma, also fails to activate T cells or produce a JSRV-specific immune response. Overproduction of surfactant proteins in OPA is also proposed to contribute to the absence of an effective immune response (71).

OPA HISTOLOGY AND COMPARISON WITH HUMAN LUNG ADENOCARCINOMAS

OPA tumors are composed of non-encapsulated neoplastic foci originating from JSRV infected and transformed bronchiolar and alveolar secretory epithelial cells (72, 73). Type II pneumocytes are the predominant cell type, with smaller numbers of bronchiolar club cells and undifferentiated cells present (74). Type II pneumocytes function to synthesize, store, and secrete alveolar surfactant, whereas bronchiolar club cells produce protein components that line the extracellular surface of bronchioles. Tumor cells are typically cuboidal or columnar, with or without cytoplasmic vacuolation while also exhibiting a low mitotic rate. However, other tumor areas may show higher degrees of malignancy with high mitotic rates and areas of necrosis (74, 75). Fibrovascular connective tissue surrounds tumor cells and acts as a scaffold for the influx of inflammatory cells. Large numbers of macrophages are typically identified (71); however, neutrophil number can vary depending on the presence of a bacterial co-infection (Figure 2). Tumor cell proliferation initially occurs along alveolar septa (lepidic growth), before extending into bronchioles through the formation of acinar or papillary proliferations. Infected cells release JSRV virions which spread within the lung forming new foci of infection, resulting

in a highly oligoclonal tumor (76). Neighboring tumor foci eventually expand and coalesce to form a single large tumor. Intrathoracic and extrathoracic metastasis is possible and has been identified in ~10% of cases (77–80).

Although early reports detailing OPA described the disease as having similarity to human BAC, under the current human lung classification system early OPA lesions would fit a description of a minimally invasive adenocarcinoma or lepidic-predominant adenocarcinoma; whereas typical advanced lesions would more closely resemble adenocarcinoma with a papillary or acinar-predominant growth pattern. Importantly, OPA has the greatest similarity to the rare multifocal, non-invasive presentation of human lung adenocarcinoma (such as the mucinous forms), and is less similar to the more common aggressive, metastatic forms of the disease (36).

EXPERIMENTAL SYSTEMS FOR STUDYING OPA

An *in vivo* sheep model was the first reproducible experimental system developed to study OPA. Initial studies showed that the injection of OPA tumor homogenates or JSRV purified from lung fluid, into the trachea of healthy sheep, led to the appearance of lung tumors (81, 82). It was later shown that using neonatal lambs improved the rate of infection and decreased the time for tumors to develop (73, 83). Further refinement of the model has been achieved through cloning and sequencing of the JSRV genome (51, 84) and the generation of an oncogenic and infectious molecular clone, which has enabled virus production using *in vitro* transfection of cell lines (85, 86). A JSRV replication-defective virus (JS-RD) that expresses only the Env glycoprotein has also been used in the *in vivo* lamb model system (55). As this vector is replication defective, it can infect and transform target cells but cannot replicate further. As these transformed cells proliferate, they form well-isolated uniform neoplastic foci, each being a separate transformed focus. Therefore, tumors induced by JS-RD have a reduced degree of polyclonality compared to naturally occurring OPA and human adenocarcinomas. This reduced heterogeneity might add value to the experimental OPA model, as the effects of targeting specific pathways would be easier to identify.

The *in vivo* lamb model also has the potential for studying pathogenic mechanisms in early stage disease. This is important as human clinical tissue from early cases is generally unavailable. However, while the lamb model is useful for studying OPA from initial infection up to the formation of small tumors, for welfare reasons it is not appropriate to let the disease reach an advanced clinical stage. As such, naturally occurring cases are more suitable for studying more advanced disease stages.

Mouse OPA models are alternative *in vivo* systems that do not necessitate the use of large animal facilities. Using both immunodeficient mice (56) and immunocompetent mice models (87) studies have shown that the intranasal administration of adeno-associated virus vectors encoding JSRV Env leads to the formation of lung adenocarcinomas that are comparable to those found in sheep and humans.

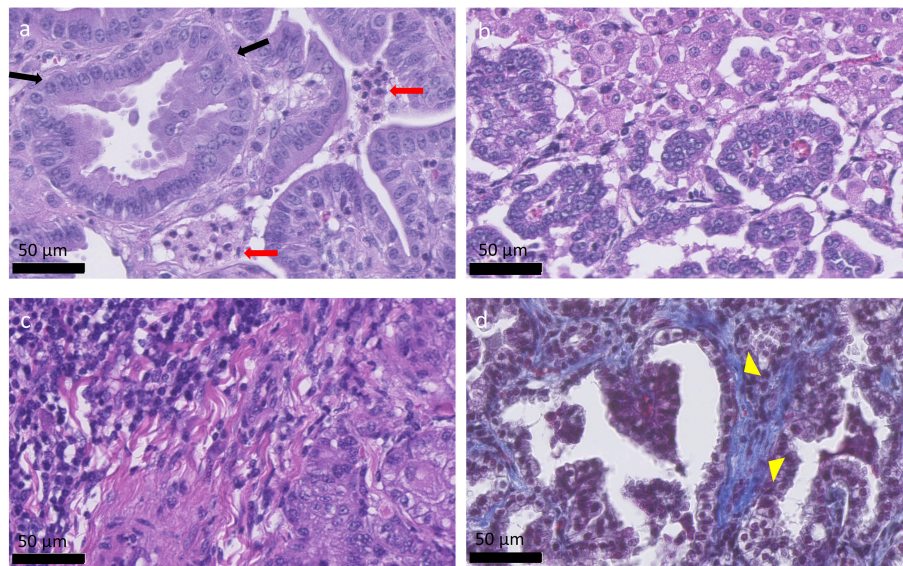


FIGURE 2 | Histological appearance of OPA tumors. **(a–c)** OPA haematoxylin and eosin stained sections. **(a)** Columnar tumor cells can be seen lining the alveolar septa (black arrows), forming acinar, or papillary proliferations. Two groups of neutrophils are present between the tumor cells (red arrows). **(b)** Alveolar macrophages can be seen at the top right-hand side of the image and are characterized by large amounts of foamy cytoplasm. **(c)** Accumulation of mononuclear immune cells, mostly lymphocytes, and plasma cells, can be observed at the top left-hand side of the image. **(d)** Masson's trichrome stained section. Collagen is stained blue and can be identified surrounding the tumor cells (yellow arrow heads), acting as a scaffold for the influx of inflammatory cells.

The lack of a cell line that can support JSRV replication *in vitro* has limited the amount of *in vitro* research that has been performed on OPA (88). Some studies have therefore focused on the use of primary OPA tumor cells (62, 89, 90); however, extended *in vitro* culture of these cells typically leads to a cessation in virus production (89, 90). These alterations in JSRV expression can be either delayed or reversed when cells are cultured in a 3D environment (89, 91), indicating that 3D culture models may more accurately recreate the oncogenic events that occur in OPA. Lung tissue explants are another *in vitro* model that has been developed. These precision-cut lung slices are tissue discs 300 µm thick and 8 mm in diameter cut using an automated microtome (59, 92), and are thought of as a transitional model between the other *in vitro* and *in vivo* available systems.

OPA AS A MODEL FOR STUDYING PULMONARY ADENOCARCINOMA TUMOURIGENESIS

It is not clear whether human pulmonary adenocarcinoma arises from a stem cell population that is able to differentiate into alveolar type II pneumocytes and bronchiolar club cells, from a lineage-specific progenitor cell, or from a fully differentiated cell type (93). In mice putative bronchioalveolar stem cells (BASC) have been identified which are proposed to be the cell type of origin of lung adenocarcinomas in response to oncogenic K-ras (94). However, the presence of BASC in humans and sheep has not been firmly established (95). Cells displaying some features of BASC have been described in sheep (72, 96) but their significance in OPA tumourigenesis remains unclear.

As described in the previous section, in the *in vivo* experimental lamb model, JSRV is able to induce the formation of OPA tumors with a short incubation period (82, 83). In contrast, adult sheep have been shown to be resistant to experimental induction of OPA (83). This age-related susceptibility to OPA tumor formation is due, at least in part, to the availability of susceptible target cells capable of being infected and transformed. JSRV, like most retroviruses, infects dividing cells much more efficiently than non-dividing cells (73). Normal sheep and human adult lungs have relatively low rates of bronchioalveolar cell division. However, the lungs of both species are not fully mature at birth and continue to develop for a period of time resulting in an increase in alveolar number (97, 98). One study has shown that the cells targeted for JSRV transformation and tumourigenesis are proliferating progenitor cells of type II pneumocyte lineage, termed lung alveolar proliferating cells (LAPCs), rather than mature post-mitotic type II pneumocytes, bronchiolar club cells, or BASC. LAPCs are significantly more abundant in lambs compared to adult sheep, therefore the age-related susceptibility of OPA development is directly related to the abundance of LAPCs (73).

The adult lung has significant reparative capabilities despite the low proliferation rate of respiratory epithelial cells, LAPCs are proposed to play an important role in tissue repair following injury. Chemically-induced injury to the respiratory epithelium has been shown to increase the number of LAPCs in adult sheep, which subsequently rendered the sheep susceptible to JSRV infection and transformation (73). This may have relevance for naturally occurring OPA, as cases typically present with a variety of other parasitic, bacterial, or viral infections (45). Classically, these infections were considered as “secondary” to

JSRV infection; however, it is possible that they are important factors that contribute to pulmonary inflammation and tissue damage that facilitate JSRV infection and tumorigenesis. In humans, recent studies have identified a subpopulation of type II pneumocytes that exhibit properties of progenitor cells, including self-renewal and proliferation in response to injury (99, 100). Thus, OPA may have value as a comparative model for understanding the role of alveolar progenitor cells in carcinogenesis.

OPA DIAGNOSIS AND POTENTIAL SOURCES OF EXPERIMENTAL ANIMALS

Although OPA has been identified in sheep <1 year old the majority of naturally occurring clinical cases are seen in sheep aged between 2 and 4 years of age. The diagnosis of clinical OPA can usually be based on clinical signs including pneumonia (non-responsive to antibiotic treatment), dyspnea, and tachypnoea (especially when herded) in combination with weight loss (despite maintaining a normal appetite) (101). Thoracic auscultation may be of benefit for diagnosing advanced cases, where adventitious lung sounds (crackles) can be heard over the majority of the lung fields due to the presence of fluid in the airways (102). Significant volumes of fluid draining from the nostrils is a pathognomonic clinical sign of OPA (103); at this stage tumors will typically occupy more than 30% of the lung volume (101). Although historically these advanced tumors were presumed to have developed over many months or years (101), new evidence shows that some OPA tumors may develop very rapidly (104).

Pre-clinical antemortem diagnosis is important not only for removing infected animals from flocks but also in identifying cases for experimental purposes; however, this diagnosis remains a significant challenge. Pre-clinical diagnosis based on a clinical examination is difficult as there may be a lack of adventitious lung sounds detectable by auscultation (105). Many infected sheep never develop clinical signs during their commercial lifespan (106), and those that do may only do so when the tumor is sufficiently large to compromise respiration. During this pre-clinical period these apparently healthy animals may be infectious and represent a source of infection for the rest of the flock.

As JSRV infected sheep fail to produce a significant humoral immune response to viral proteins (107), it has not been possible to develop serological diagnostic assays. Alternative diagnostic tests have been developed for virus detection in blood samples using PCR technology (108); unfortunately the numbers of virally infected blood mononuclear cells (monocytes, B and T lymphocytes) are very low, which results in high false negative results (109). Despite this significant limitation, the test can be used for identifying infected flocks rather than for testing individual animals. The same PCR technique has been employed to detect JSRV-infected cells in bronchoalveolar lavage samples (110), which offers better sensitivity than the blood test. However, this method requires sedation for sample collection, only tests a small region of

the lung (potential for missing early cases) and does not lend itself to large-scale routine on-farm testing. Currently, the gold standard diagnostic test for both clinical and pre-clinical OPA remains gross pathology and histology performed at post mortem examination. OPA tumors can be extensive, involving the entire lung lobe, or may occur as multifocal discrete lesions. These lesions fail to collapse upon entering the thoracic cavity and can distort the normal architecture of the affected lung lobe, with clear boundaries between tumor tissue and adjacent pink aerated lung. Although the overlying pleura can remain intact, fibrinous adhesions between the visceral pleura and chest wall can be seen (Figure 3). Tracheobronchial and mediastinal lymph nodes usually appear grossly normal but may be enlarged in cases of metastasis or pneumonia (39).

Imaging modalities such as radiography and computed tomography (CT) have been suggested for use in OPA diagnosis. CT is considered the gold standard imaging modality for human lung parenchyma and has been used in studies to monitor the development and progression of OPA in both naturally occurring (111) and experimentally infected animals (70). CT will detect smaller lung lesions than can be identified using radiography, particularly if located in the ventral margins of the cranial lung lobes that are difficult to image using radiography (Figure 4). However, radiography and CT are cost prohibitive for commercial flocks and require specialized equipment and sedation/general anesthesia (101). Ultrasonography is an extremely useful imaging technique for OPA diagnosis and can be performed on-farm in conscious animals. With experience, the procedure can be performed in <1 min per sheep (112), can differentiate between chronic lung lesions and can detect OPA lesions as small as 1–2 cm in diameter involving the visceral pleura (31). One study conducted transthoracic ultrasound examinations of 100 sheep presented for the investigation of weight loss with or without respiratory signs; of these cases, 41 sheep were diagnosed as OPA positive based on ultrasound examination alone, with all cases having the diagnosis confirmed at post mortem. The remaining sheep had no ultrasonographic changes characteristic of OPA and had no gross OPA lesions at post mortem. The study demonstrated the high specificity of transthoracic ultrasound for OPA diagnosis in clinically affected animals, producing no false positive or negative results (31). Although a negative scan cannot guarantee that an animal does not have early OPA and/or is not infected with JSRV, it has been suggested that transthoracic ultrasound examination can be used to confirm a suspected diagnosis, screen flock replacements, and screen sheep in known OPA-affected flocks. It is also an ideal method for identifying pre-clinical cases for experimental use, as individual cases can be selected based on the size and location of OPA lesions.

OPA AS A PRE-CLINICAL MODEL

The use of OPA as a model for monitoring the tumor microenvironment, assessing the effectiveness of chemo-

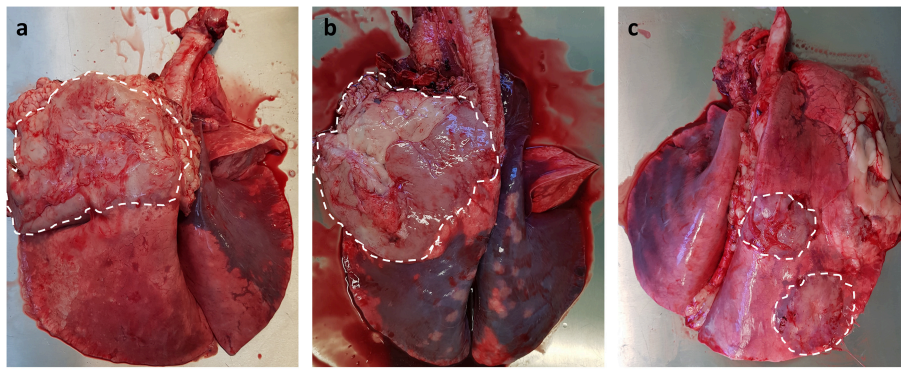


FIGURE 3 | Gross pathology of OPA tumors. **(a,b)** Large single advanced OPA tumors affecting the entire left cranial lung lobe. The lesions are gray in color with a clear distinct boundary between neoplastic tissue and the neighboring pink aerated lung. Extensive fibrous tissue can be seen attached to the overlying pleura of the tumor. **(c)** Two discrete OPA tumors within the right cranial and caudal lung lobes.

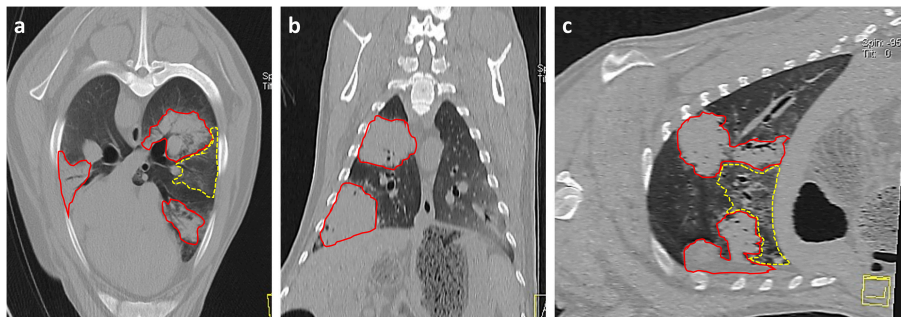


FIGURE 4 | Thoracic CT images of OPA tumors. **(a)** Axial, **(b)** Coronal, and **(c)** Sagittal planes. Three large areas of increased radiopacity are seen within the lung lobes consistent with advanced OPA tumors (outlined in red). One lesion is present within the dorsal region of the left cranial lung lobe with a further lesion in the ventral region of the left caudal lung lobe. A smaller lesion is present within the right caudal lung lobe. A patchy and hazy area of increased opacity (ground glass appearance), with preservation of bronchial and vascular patterns, is present (outlined in yellow) between the two tumors in the left lung lobes. This area is consistent with regions of neoplastic foci or a secondary pneumonia.

and radiotherapy or in the development of surgical techniques has not been previously documented. However, if techniques that are commonly used in the treatment of human lung cancer patients such as ultrasound, general anesthesia, CT, and surgery can be incorporated into the OPA model, this would further demonstrate its potential as an excellent translational research tool. One paper documented the use of naturally occurring OPA cases combined with CT evaluation, post mortem examination/histopathology, trace element, and liver enzyme activity analysis in a long-term study evaluating the impact of nutritional selenium on tumorigenesis and progression (111). This study demonstrated the potential for the OPA model to be integrated with multiple techniques to provide comprehensive information on tumor pathogenesis.

In terms of chemotherapy models, *in vitro* work using rat fibroblasts has shown that through AKT degradation, Hsp90 inhibitors can block the transformation and revert the phenotype of cells already transformed by JSRV Env. Hsp90 inhibitors can also reduce the proliferation

of primary and immortalized OPA cell lines (63). The chemotherapeutic potential of agents such as Hsp90 inhibitors could be assessed using OPA cases if techniques could be integrated into the model to assess the tumors response to treatment.

One current ongoing multidisciplinary project that is using naturally occurring OPA cases as a pre-clinical translational model is the Implantable Microsystems for Personalized Anti-Cancer Therapy (IMPACT) programme at the University of Edinburgh (113). This project aims to develop novel miniaturized implantable oxygen and pH sensors that can monitor oxygen levels and pH within a solid tumor; the identification of hypoxic and acidic regions within a tumor can lead to more targeted therapies against these radiation and chemo-resistant regions. Functionality of these sensors is being validated following their implantation into OPA tumors using a CT-guided percutaneous method. This technique is similar to that used for transthoracic needle biopsies in human patients. If successful, then studies such as this will provide exciting new translational opportunities for the OPA model to be used in

pre-clinical research (see accompanying article, Gray et al. manuscript submitted)¹.

CONCLUSION

As outlined here, OPA has great potential to be used as an excellent model for studying multiple aspects of human lung cancer biology. As a result, *in vivo* and *in vitro* OPA experimental models have been developed for the study of JSRV Env mediated oncogenesis; these have been successfully used to determine the molecular pathways involved in lung cancer pathogenesis. However, the potential for OPA to be used as a pre-clinical animal model for assessing human lung cancer treatment strategies has yet to be fully exploited. Naturally occurring OPA cases are readily available from infected flocks due to the endemic nature of the disease in many countries and pre-clinical cases can be identified by the use of ultrasound scanning programmes. The use of naturally occurring cases could decrease the use of experimentally induced OPA tumors in lambs, reducing ethical concerns with

this model. Future studies that can integrate techniques commonly used in the treatment of human lung cancer patients, such as ultrasound, general anesthesia, CT, and surgery, would further strengthen the effectiveness of OPA as a pre-clinical cancer research model.

AUTHOR CONTRIBUTIONS

MG wrote the majority of the manuscript and composed the majority of the figures with contributions from JM who wrote the experimental systems for studying OPA. Critical revisions were made by MG, JM, PS, JRKM, SG, RG, RC, CW, CC, DG, AM, and DA. All authors read and approved the final manuscript.

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¹Gray M, Sullivan P, Marland JRK, Greenhalgh SN, Meehan J, Gregson R, et al. A novel translational ovine pulmonary adenocarcinoma model for human lung cancer.

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Radiologic Modalities and Response Assessment Schemes for Clinical and Preclinical Oncology Imaging

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Clinical drug trials for oncology have resulted in universal protocols for medical imaging in order to standardize protocols for image procurement, radiologic interpretation, and therapeutic response assessment. In recent years, there has been increasing interest in using large animal models to study oncologic disease, though few standards currently exist for imaging of large animal models. This article briefly reviews medical imaging modalities, the current state-of-the-art in radiologic diagnostic criteria and response assessment schemes for evaluating therapeutic response and disease progression, and translation of radiologic imaging protocols and standards to large animal models of malignant disease.

Keywords: Radiology, imaging, trial, standards, large animal model

INTRODUCTION

Clinical trials are an essential component of the drug development process, and are used to aid in the systematic assessment of newly developed agents. This regulatory framework was developed in order to create a standardized methodology for evaluating drug safety and efficacy. Given that a clinical trial for the development of a new drug requires an investment of 10 years and well over \$1 billion—with greater cost to drug developers and public health risk to patients should a drug go to market and subsequently be deemed unsafe or ineffective—drug developers are eager to optimize trials in order to improve early identification of drug failure and minimize confounding variables, biases, and statistical errors (1). Outcome measures used to inform decision-making vary depending on trial phase, whereby early phases evaluate safety and potential efficacy of a drug, and later phase trials assess effect on clinical outcomes compared to a placebo or standard of care. Regardless of trial phase, radiologic imaging has emerged as a valuable tool for assessment of drug efficacy in oncology clinical trials due to the ability to longitudinally assess tumor size and viability in a non-invasive and standardized manner. The two-fold purpose of this review article is to: (1) provide an overview of medical imaging and tumor response assessment standards for clinical oncology trials in order to provide necessary context on imaging needs for preclinical cancer trials and to offer example systems from which to develop animal based imaging standards and schemes in the future; and (2) to describe the applicability and translation of radiologic imaging protocols and standards to preclinical large animal models of malignant disease, with a focus on unmet needs.

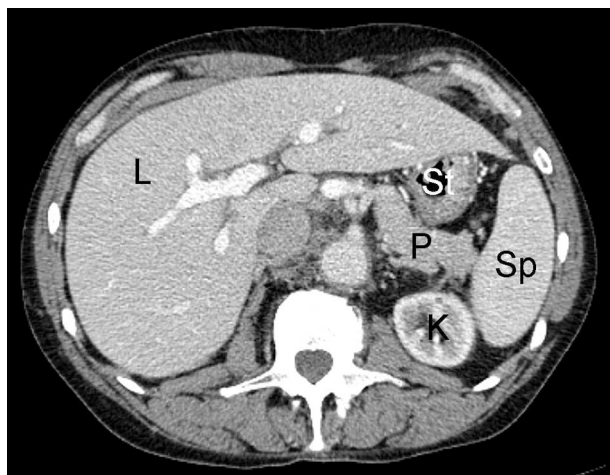


FIGURE 1 | Example of normal contrast-enhanced abdominal CT scan in human patient; L, liver; K, kidney; P, pancreas; St, stomach; Sp, spleen.

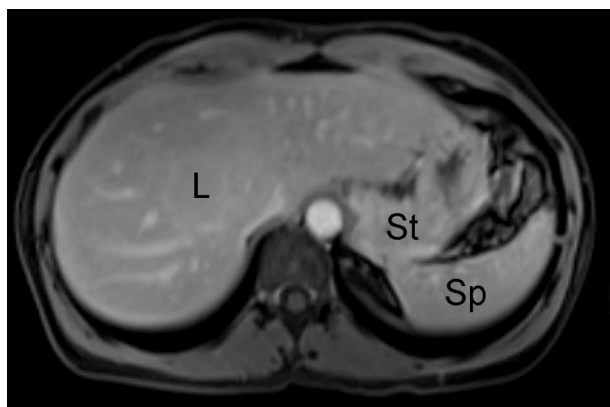


FIGURE 2 | Example of unremarkable contrast-enhanced abdominal MR imaging study of the abdomen in human patient; L, liver; St, stomach; Sp, spleen.

IMAGING MODALITY OVERVIEW

A synopsis of radiologic imaging modalities relevant to oncology clinical trials—spanning computed tomography (CT), magnetic resonance (MR) imaging, ultrasound, and positron emission tomography (PET)—is presented in **Table 1**.

Computed Tomography

Introduced in 1972, CT was the first non-invasive radiologic imaging technique allowing for tomographic imaging without superimposition of neighboring anatomic structures onto one another. This imaging technology operates through the acquisition of x-ray images spanning different angles across a single axis of rotation, and uses computer algorithms to reconstruct these planar projection images into cross-sectional slices (**Figure 1**). The x-ray imaging technology which this imaging modality is based upon measures the attenuation

of high energy photon beams transmitted through a subject. Measurement of the attenuation coefficient allows for the differentiation of tissues based on their density, as tissues with high density (such as bone) will attenuate a higher proportion of a photon beam than those with lower densities (such as muscle or fat). Detecting these subtle differences in tissue density is helpful in the detection of tumors, as disordered neoplastic growth may result in changes in tissue density (2). Modern CT imaging has many advantages, including the ability to image large volumes with sub-millimeter resolutions in a short time span, and the capability for multi-planar reformatting of images in sagittal and coronal views after imaging data has been acquired. CT imaging does carry small risk, however, as the exposure to high doses of ionizing radiation may increase the probability of developing some cancers (3–5). However, advances in CT instrumentation, detector technologies, and image reconstruction algorithms have allowed for the acquisition of high quality images with significant radiation dose reduction (6–8).

MR Imaging

MR imaging is a widely available imaging technique that uses a magnetic field and radiofrequency pulses to non-invasively generate cross-sectional images using the inherent magnetic properties of the human body. Initially applied to human imaging in 1977, this modality utilizes various pulse sequences, which are time varying gradient magnetic fields coordinated with radiofrequency pulses. These pulse sequences take advantage of tissue specific properties of magnetic relaxivity (termed T1 and T2) in order to generate image signal and contrast (**Figure 2**). These pulse sequences can be implemented in a variety of ways, and can be used to selectively null signal from fat and to measure properties such as the diffusion of water within a tissue, among numerous other applications. Unlike CT, MR imaging employs radiation in the radiofrequency range which is non-ionizing, making it more suitable for repeat imaging sessions. MR imaging does have limitations, however, as it carries risk for patients with metallic implants such as pacemakers, synthetic valves, orthopedic prostheses, and aneurysm clips due to the possibility of dislodging these implants from interaction with and motion because of the magnetic field (9). There is also the risk of heating of tissues adjacent to implants due to deposition of radiofrequency energy (10). Another disadvantage of MR imaging is the lengthy imaging time required to conduct high resolution MR imaging protocols, which can increase the possibility of patient motion and lead to image quality degradation. However, the adoption of image acceleration techniques (such as parallel imaging and compressed sensing) have allowed for reduction of these long scan times to more reasonable durations (11, 12).

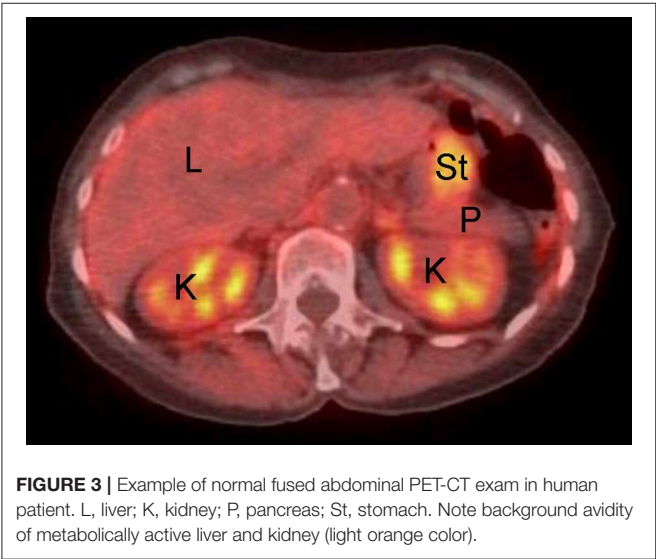
Ultrasound

Ultrasound is a medical imaging modality that uses high-frequency sound waves to generate images. This technique uses a piezoelectric transducer, which transmits sound waves by converting electrical energy into mechanical energy in the form of vibration. This transducer can also detect reflections of these transmitted sound waves which occur when the

TABLE 1 | Overview of radiologic imaging modalities relevant to oncology clinical trials.

Parameter	CT	MR imaging	PET
Imaging basis	Ionizing x-radiation	Non-ionizing radiofrequency radiation	Ionizing radiation (positron emitting radiotracer ± x-radiation for concurrent low dose CT scan)
Preparation	None needed	None needed	Fasting × 6 h (diabetic patients require close glucose monitoring); no strenuous activity before study to avoid muscular deposition of radiotracer;
Contraindications	Avoid in pregnant women and pursue with caution in women of childbearing age	Avoid in patients with metallic implants (e.g., pacemakers, aneurysm clips)	If concurrent low dose CT scan obtained, avoid in pregnant women and pursue with caution in women of childbearing age
Acquisition time	Short (5 min)	Long (30–90 min)	Long (60 min for radiotracer to reach target tissue + 30 minutes for imaging)
Contrast material	Iodinated contrast material; may cause contrast-induced nephropathy	Gadolinium contrast material; may cause nephrogenic systemic fibrosis	None

CT, computed tomography; MR, magnetic resonance; PET, positron emission tomography.



ultrasound enters media of different acoustic impedance. Acoustic impedance is a physical property of a material, defined as the resistance for the propagation of sound waves which varies as a function of the material density. These reflected waves—termed “echoes”—form the basis of image generation with ultrasound (13). When ultrasound waves travel through tissue with high acoustic impedance, a large amount of the incident acoustic energy is reflected and the tissues appear bright, or hyperechoic. On the other hand, ultrasound waves traveling through a tissue with low acoustic impedance results in greater transmission and less reflection of the incident energy, producing tissue that appears dark or hypoechoic.

Due to its low cost and relative accessibility compared to other medical imaging modalities, ultrasound has become a widely-adopted imaging tool used to screen for cancer, vascular disease,

and trauma (14–16). It is also commonly used to aid with many image-guided procedures performed in real time. However, ultrasound is rarely used for longitudinal follow-up of diseases such as cancer, as the high degree of operator dependence and variability in image acquisition may result in underestimation of tumor size. This is due to the potential variations in imaging technique precluding consistent imaging for capture of maximal disease dimensions (17). For this reason, human clinical trials employ other cross-sectional imaging techniques such as CT and MRI, which are highly reproducible and less operator dependent than ultrasound.

PET Imaging

Popularized in 1990, PET is another imaging modality that has come into widespread use in clinical trials due to its ability to evaluate tumor metabolic response to therapy. Fluorine-18 (¹⁸F)-fluorodeoxyglucose (FDG) is a positron-emitting isotope which has become prevalent as a metabolic marker for imaging cancers of various origins (although this is not the only employed PET agent). As FDG is structurally similar to glucose, this radiotracer is taken up by cells much like the unlabeled sugar and undergoes the first step of glucose metabolism. After this step, however, the phosphorylated FDG molecule is trapped within a cell and, for all practical purposes, is not metabolized further. Given cancer cell preference for glycolysis as an energy source, this radiotracer and associated imaging technique allow for localization of neoplasms and metastatic disease that are highly glucose avid. While FDG is an effective tumor localizing agent, it should be noted that it is not specific for tumors alone, in that tissues with high background glucose uptake or excretion, such as the brain, kidneys, heart, and muscle, as well as inflamed tissues, may also exhibit high FDG signal. It should also be noted that not all tumors are FDG avid, and while some cancer types consistently exhibit moderate-to-high uptake, others are variable in their uptake, making the utility of this modality tumor-specific (18). The major advantage of PET imaging is the

ability to image tissue viability rather than merely anatomy or structure; this provides useful functional oncologic information to guide clinical decision making. The main disadvantage of PET is its relatively low spatial resolution, a limitation which has been overcome in part through the co-registration of PET images with low dose CT images (termed “PET-CT”) to allow for better anatomic delineation of PET imaging observations (**Figure 3**). Recently, PET-MR imaging—which merges the tissue sensitivity and quantitative imaging features of MR imaging with the physiologic information of PET—has been investigated for its multimodal radiologic imaging capabilities (19).

RATIONALE FOR USE OF MEDICAL IMAGING IN CLINICAL TRIALS

Contemporary medical imaging modalities are critical to the assessment of drug efficacy in oncology clinical trials. The non-invasive nature of radiologic imaging allows for serial monitoring of tumor stage throughout the treatment period, which, unlike more invasive tissue- or blood-based assays, avoids unnecessary patient trauma and allowing for use at more frequent intervals. Furthermore, clinically useful surrogate trial endpoints such as time-to-progression (TTP) and progression free survival (PFS) can be assessed by imaging, and have come into frequent use in drug trials as they may be observed shortly after initiation of therapy, and allow for an early assessment of treatment response; in contrast, use of overall survival (OS) as a primary endpoint requires protracted trial lengths to achieve, as well as relatively larger patient population required to properly power a study (20). These radiologic imaging outcome measures can thus help to reduce length and cost of trials, and also may allow trials to be adequately powered with smaller numbers of subjects, though

these surrogate endpoints must be validated and demonstrated to be tightly correlated with clinical endpoints (21–23).

Additional benefits of medical imaging in clinical trials include image quantitation, automated processing and measurements, and real time transmission from trial sites to contracted research organizations that evaluate trial data. Quantitative measurement of medical imaging increases the accuracy of interpretation by eliminating subjective assessment of data, and the advent of automated and semi-automated image processing pipelines can reduce reader variability in trial analyses. The evaluation of large scale multicenter trial data requires rigorous standardization, in order to allow evaluation of patient data both longitudinally and across multiple sites. Variability can occur in both image acquisition and image interpretation, and so it is essential that standards are set a priori to minimize variation introduced by differences in scanner hardware, imaging parameters, contrast agent type and administration, or patient positioning. Thus, standards have been put in place, spanning regular calibration of scanners with phantom studies in order to account for performance drift, proper training of technologists to maintain consistency in patient positioning and acquisition, as well as proper blinding of readers in order to reduce bias (24).

DIAGNOSTIC SCHEMES AND RESPONSE ASSESSMENT CRITERIA: EXAMPLES FROM HUMAN CLINICAL CARE

In addition to rigorous standardization for image acquisition, standards must be set for interpretation of medical imaging data in order to enhance reporting consistency, reduce inter- and intra-reader variability, and increase comparability across investigations. For this reason, diagnostic schemes and response assessment criteria have been created to report findings using a systematic methodology and to provide universal descriptive verbiage such that reporting may be objective and reproducible regardless of reader.

Diagnostic Schemes

Diagnostic classification systems allow for reliable and systematic interpretation of radiologic imaging studies (25). The American College of Radiology (ACR) supports several such schemes, including breast (BI-RADS), prostate (PI-RADS), and liver (LI-RADS) Imaging Reporting and Data System schemes, among others (19). Using liver imaging as an example, the ACR LI-RADS was developed in 2011 as a comprehensive classification system which standardizes radiological interpretation for liver cross-sectional imaging in patients at risk for primary liver cancer, or hepatocellular carcinoma (HCC) (26). This 5-point scale reporting system uses major and minor imaging features to classify a liver abnormality as definitely (LI-RADS 1) or probably (LI-RADS 2) benign, intermediate probability of malignancy (LI-RADS 3), probably malignant (LI-RADS 4), or definitely malignant (LI-RADS 5) (**Figure 4**). The classification of liver abnormalities is performed using features such as size, interval growth, arterial phase hyper enhancement, portal venous phase



FIGURE 4 | Arterial (**Left**) and venous (**Right**) phases of contrast enhanced CT scan performed in human patient demonstrate typical LI-RADS 5 mass (arrow), displaying typical arterial phase hyper enhancement, venous phase “washout,” and enhancing capsule.

TABLE 2 | Response outcome definitions for response assessment schemes.

Measurement guidelines	WHO	RECIST 1.1	EASL	mRECIST	PERCIST
Measurement guidelines	Change in sum of products of maximum bi-dimensional tumor diameters	Change in sum of maximum uni-dimensional tumor diameters; measurement of 5 target tumors with maximum of 2 tumors per organ	Change in sum of products of maximum bi-dimensional tumor diameters of viable (enhancing) tissue	Change in sum of maximum uni-dimensional tumor diameters of viable (enhancing) tissue; maximum of 2 liver tumors measured	Change in maximum SUL (SUV corrected for lean body mass); minimum target SUL should be > 1.5x mean liver SUV + 2x SD liver SUV; target tumor with peak SUL at follow-up may be different from that selected at baseline
CR	Disappearance of all measurable tumors	Disappearance of all measurable tumors; short axis of all pathologic lymph nodes <10 mm	Disappearance of all intra-tumoral enhancement in target tumors	Disappearance of all intra-tumoral enhancement in target tumors	Normalization of all target and non-target tumors to less than mean liver SUL, and equal or less than normal surrounding tissue
PR	≥50% reduction in sum of cross-products	≥30% reduction in sum of maximum diameters	≥50% reduction in sum of cross-products of viable (enhancing) tissue	≥30% reduction in sum of cross-products of viable (enhancing) tissue	≥30% reduction in peak SUL; absolute change must be ≥0.8 SUL
SD	Neither PR nor PD	Neither PR nor PD	Neither PR nor PD	Neither PR nor PD	Neither PR nor PD
PD	≥25% increase in sum of cross-products	≥20% increase in sum of cross-products	≥25% increase in sum of cross-products of viable (enhancing) tissue	≥20% increase in sum of cross-products of viable (enhancing) tissue	≥30% increase in peak SUL; absolute change must be ≥0.8 SUL

WHO, World Health Organization; RECIST, Response Evaluation Criteria in Solid Tumors; EASL, European Association for the Study of the Liver; mRECIST, modified Response Evaluation Criteria in Solid Tumors; PERCIST, Positron Emission Tomography Response Criteria in Solid Tumors; CR, complete response; PR, partial response; PD, progressive disease; SD, stable disease; SUV, standardized uptake value; SUL, SUV lean.

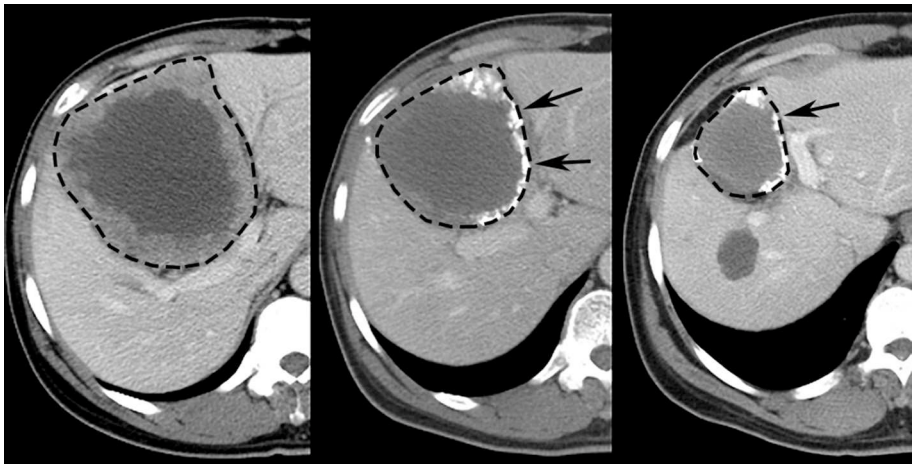


FIGURE 5 | Representative images displaying RECIST response after TACE treatment of HCC in human patient. Pretreatment (**Left**) contrast-enhanced CT scan depicts 10.6 cm diameter right lobe liver tumor (dashed line). Sequential post-treatment contrast-enhanced CT scans (**Middle, Right**) reveal ensuing size reduction to 7.5 cm (29% reduction, RECIST SD) and 5.0 cm (53% reduction, RECIST PR) diameter, respectively; high attenuation material at tumor periphery (arrows) represents chemotherapy emulsion.

hypo enhancement, and capsular enhancement as major criteria, the presence of which favors the likelihood of malignancy. Utilization of LI-RADS enables the radiologist to employ specific descriptive terminology for consistent radiological reporting of liver abnormalities to meaningfully guide follow-up and/or treatment (27).

Response Assessment Criteria

Response assessment schemes similarly allow for consistent and systematic interpretation of tumor response to treatment on radiologic imaging studies. A summary of various response assessment systems used in clinical oncology trials is presented in **Table 2**.

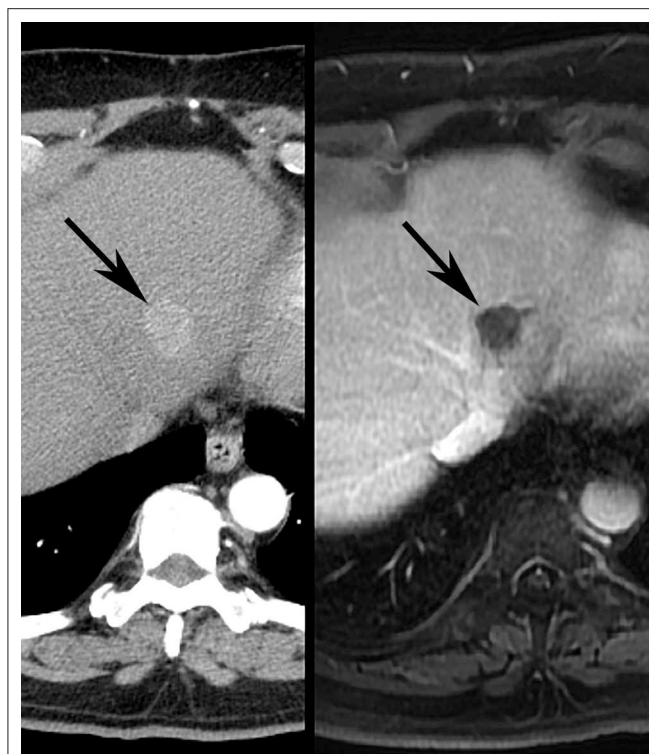


FIGURE 6 | Illustrative images demonstrating EASL response after TACE treatment of HCC in human patient. Pretreatment **(Left)** contrast-enhanced CT scan depicts 2.0 cm diameter left lobe liver tumor (arrow). Post-treatment contrast-enhanced MR imaging study **(Right)** shows no residual enhancing component (arrow), consistent with EASL CR.

The original response criteria, first outlined by the World Health Organization (WHO) in the early 1980s, were anatomic in nature and based on the sum of the products of maximal perpendicular linear measurements of tumors. This guideline for response assessment has since been replaced by the Response Evaluation Criteria in Solid Tumors (RECIST)—created in 2000 and revised in 2009—which utilizes maximal unidimensional measurements and addresses some of the pitfalls and limitations of the original WHO guidelines. Although these response assessment criteria were generated during the era of cytotoxic chemotherapeutic agents, both remain in widespread use in clinical trials—with RECIST criteria in most widespread use in trials (28, 29)—and can be effective in situations where successful therapy results in a reduction in tumor size (30–33) (**Figure 5**).

With the advent of new interventions such as immunotherapy and agents that selectively modulate specific molecular targets, it has become clear that tumor size changes are not the only or even the most effective indicator of treatment response for all cancer therapeutics. In patients with HCC, for example, locoregional therapies such as transarterial chemoembolization (TACE) induce tumor necrosis, often without change in tumor size (34–38). As such, a panel of HCC experts organized by the European Association for the Study of the Liver (EASL) generated a new set of response criteria which would take tumor necrosis into account. These EASL criteria would use reduction

in viable tumor area, as determined by contrast enhancement on contrast-enhanced CT and MR imaging, as the primary method for evaluating treatment response in HCC (**Figure 6**). This was followed by a formal amendment to RECIST criteria in 2010—termed mRECIST—which would draw from the EASL definition of viable tumor, and simplify the measurement system from EASL criteria by using unidimensional linear summation (39, 40) (**Figure 7**). Several studies have since confirmed that both the EASL and mRECIST schemes may be better predictors of survival than WHO and RECIST criteria, respectively, for certain cancers (41–45), and may demonstrate better correlation with pathologic necrosis (46).

FDG-PET radionuclide imaging has long been considered a potentially useful tool in detection of subclinical response to anti-tumor therapies. However, this technique also poses unique challenges in standardization of acquisition and reporting of results. The European Organization for Research and Treatment of Cancer (EORTC) proposed a common method for image acquisition, measurement of radiotracer uptake, and reporting of response data (47). The Positron Emission Tomography Response Criteria in Solid Tumors (PERCIST) scheme was later developed in 2009 and sought to further standardize the assessment of tumor metabolic response, and described detailed methods to allow longitudinal comparison of PET images (**Figure 8**). The PERCIST criteria utilizes a different method for image interpretation, and adds reporting instructions to clarify the time of imaging relative to initiation of therapy, as tumor radiotracer uptake can vary temporally depending on time from therapy (48, 49).

While EORTC and PERCIST have differences in implementation, the two criteria have demonstrated excellent agreement (50). Several studies have demonstrated that rapid reduction in FDG uptake in tumor, seen shortly after therapy, was correlated with later pathologic and radiographic response, with PET response far preceding any reduction in tumor size, while increases, no change, or modest reductions in FDG uptake after initiation of therapy were more likely to portend non-response (51–53). Importantly, studies have demonstrated that tumors exhibiting a partial metabolic response after initiation of therapy (as measured by PERCIST) were correlated with longer TTP and longer OS than those tumors that exhibited persistently high FDG uptake (54–57), and highlight the potential value of FDG-PET imaging and standardized metabolic response criteria such as PERCIST as a means for early identification of responders to therapy (58). Notably, combining anatomic imaging modalities (e.g., CT) with functional imaging data (e.g., PET) has shown value in assessing tumor response to therapy by leveraging both tumor size and metabolic changes toward optimal assessment of tumor response (59, 60).

ANIMAL IMAGING FOR PRECLINICAL ONCOLOGY TRIALS

Challenges in Animal Imaging

All of the described radiologic modalities may be used for imaging in preclinical animal models of disease. However, animal

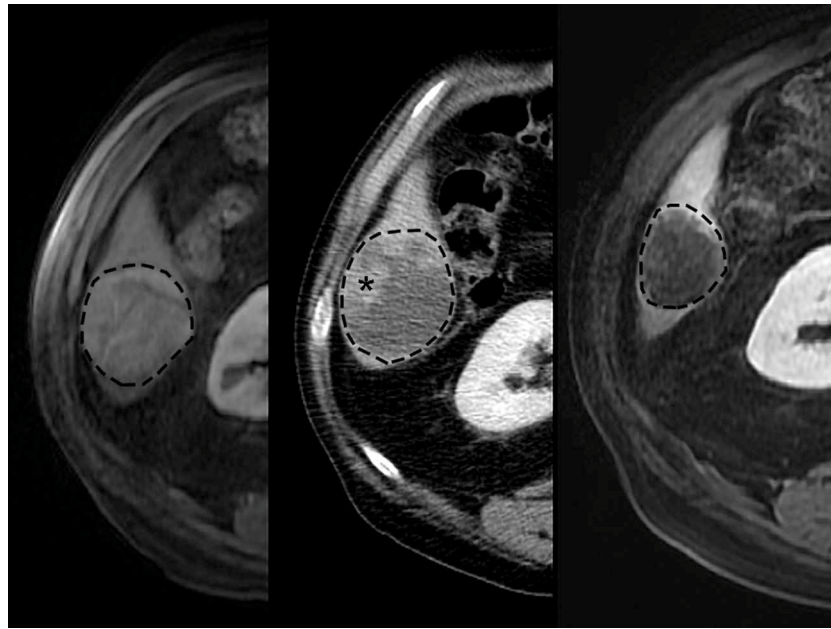


FIGURE 7 | Typical images displaying mRECIST response after TACE treatment of HCC in human patient. Pretreatment (**Left**) contrast-enhanced MR imaging exam depicts 5.0 cm diameter right lobe liver tumor (dashed line). Contrast-enhanced CT scan (**Middle**) after first treatment demonstrates 1.5 cm residual enhancing tumor (asterisk) (70% reduction, mRECIST PR). Retreatment pursued, and contrast-enhanced MR imaging scan (**Right**) after second treatment demonstrates no residual enhancing tumor (100% reduction, mRECIST CR).

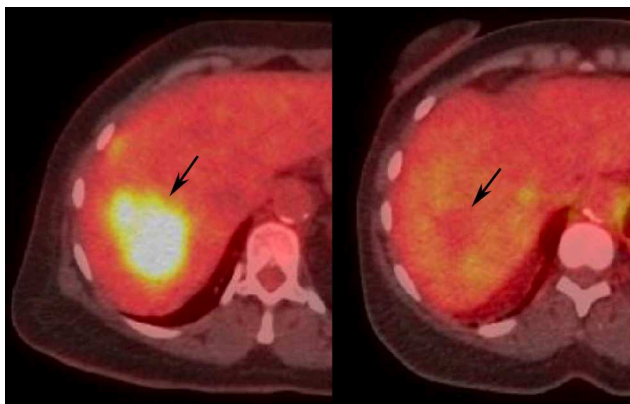


FIGURE 8 | Illustrative images demonstrating PERCIST response after locoregional treatment of liver tumor in human patient. Pretreatment (**Left**) PET-CT demonstrates FDG avid liver tumor (arrow). Post-treatment (**Right**) PET-CT shows normalization of SUL (arrow), consistent with PERCIST CR.

radiologic imaging primarily differs from human clinical imaging in regards to the need for anesthesia; while human subjects are primarily imaged awake, animals are generally imaged under anesthesia to reduce gross motion during image acquisition. While examples exist of animals being trained to tolerate imaging procedures under intravenous sedation (61), as well as stereotactic techniques for restraining the body and head of smaller animals, longer imaging procedures such as MR imaging and PET acquisitions generally require anesthesia to prevent

motion related image degradation. The use of anesthesia poses a unique set of challenges, and adds a layer of complexity for standardization in terms of animal handling, monitoring, and reporting. For instance, the reduced cardiac and respiratory drive caused by many anesthetics necessitates constant physiological monitoring (62). This becomes a logistical challenge inside an MR imaging suite, where neither radiofrequency emitting electronics (given risk for imaging artifacts) nor ferromagnetic materials (given risk for susceptibility artifacts, dislodgement, or near field heating) can be used. In addition to logistical challenges with monitoring during image acquisition, anesthetic agents can have variable effects on physiology, such as cardiac and respiratory depression, changes in cerebral blood flow and volume, and alterations in body temperature. While these physiological derangements may not affect structural imaging, they have been shown to affect radiotracer distribution and bioavailability, confounding the results of metabolic imaging modalities such as FDG-PET. In all, these considerations demonstrate the need for reporting of anesthetic agents used as well as animal handling protocols, as these factors can affect and confound imaging results (63–65).

EXISTING RESPONSE ASSESSMENT SCHEMES FOR ANIMAL CLINICAL ONCOLOGY TRIALS

With the growing use of large animal models of cancer, and the increasing number of prospective clinical trials using such

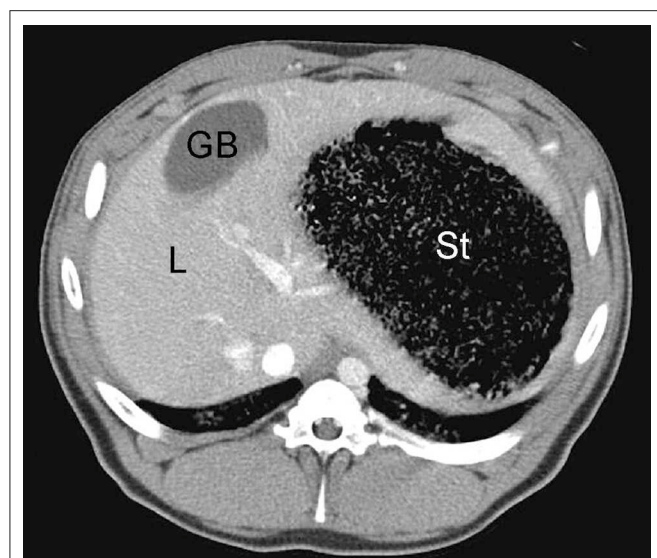


FIGURE 9 | Example of normal contrast-enhanced porcine abdominal CT scan; L, liver; GB, gallbladder; St, stomach.

models for assessing response to various therapies, there is an emerging need to standardize methodologies for evaluating tumor response in large animal models order to both improve the accuracy and consistency of reporting between various treatments and studies and increase the translatability to human clinical care. Currently, there is a paucity of published response evaluation criteria directly applicable in animal model systems. Given the lack of formal guidelines for assessment of response to therapy for solid tumors in animal models, the Veterinary Cooperative Oncology Group (VCOG) generated a consensus document based on recommendations from a subcommittee of the American College of Veterinary Internal Medicine (ACVIM) board certified veterinary oncologists in order to facilitate the design of a standardized protocol that would provide consistent, accurate, and reproducible reporting in therapeutic trials using animal oncology models. To that end, VCOG used the commonly implemented human response evaluation criteria RECIST as a framework for creating the canine response evaluation criteria in solid tumors (cRECIST v1.0), which is meant to provide specific guidelines for the measurement of solid tumors before, during, and after the initiation of therapy in prospective clinical trials using canine solid tumor models. This methodology is meant to be easily implemented, reproducible, and if widely adopted as anticipated, will standardize response assessment protocols to enable the comparison of current and future treatment strategies.

Recommendations from cRECIST follow many of the guidelines laid out from clinical RECIST. Some of these include baseline measurement of a tumor as close to the initiation of treatment, but no greater than two weeks prior to start of treatment. As with RECIST, the longest diameter in the plane of measurement should be recorded, and all subsequent measurements should be performed in the same plane of measurement. The minimum size of target tumors is 10 mm, and

those masses falling below this threshold in the longest plane are considered non-measurable. If there exists more than one measurable target mass, a maximum of five target tumors should be reported with a maximum of two tumors per organ. Non-measurable and non-target tumors may be used in assessing overall tumor burden and should be reported as “present” or “absent” on follow-up, however for studies where tumor response is the endpoint, only subjects with measurable disease may be included. For studies where progression is the endpoint, the protocol must state whether subjects with non-measurable disease may be included. Much like RECIST, assessment of lymph nodes should report the longest diameter along the greater of either width or height at baseline (not length), and use a minimum size of 15 mm along this axis of measurement.

In terms of image acquisition, cRECIST recommends CT as the preferred imaging modality over MR imaging. This is due to the greater reproducibility in measurements; however, either may be used for measurement of tumors. Indeed, MR imaging provides superior soft tissue contrast, however the rapid image acquisition of CT paired with its high spatial resolution results in reduced motion during image acquisition and improved delineation of tumor boundaries, respectively, which are the likely reasons for the improved measurement reproducibility in CT. Ultrasound is generally not recommended due to the potential variability in acquisition, but given the cost of CT and MR imaging as well as the need for anesthesia (to reduce motion), cRECIST provides suggested guidelines for the use of ultrasound. These suggestions recommend that the same user perform assessments using the same machine at each time point in order to reduce inter-observer variation, a minimum target tumor diameter of 20 mm at baseline, and use of previously documented images to serve as a guide for subsequent imaging in order to allow for reassessment using previously used planes of image analysis (66).

While the canine cRECIST system represents a concrete example of standardization of imaging response assessment in animals, dogs represent clinical veterinary patients rather than biomedical animal model systems, and the translatability of the cRECIST scheme to biomedical animal models is unclear. At present, there are no available response evaluation criteria strategies for use in other large animal species, such as pigs. This fact is substantiated by the wide variation of response assessment methods used in published preclinical investigations (67), which span simple reporting of tumor diameter to description of percent tumor growth or involution, and which lack a common language for comparison across published studies. Development and validation of standardized tumor response assessment systems applicable in biomedical animal models represents an important barrier to broad employment of large animals in preclinical trials, and one which must be overcome if radiologic imaging is to be utilized for preclinical trial applications.

Unmet Medical Imaging Needs for Large Animal Clinical Oncology Trials

While human clinical trials are the benchmark for advancing standard-of-care cancer therapeutics, the regulatory and

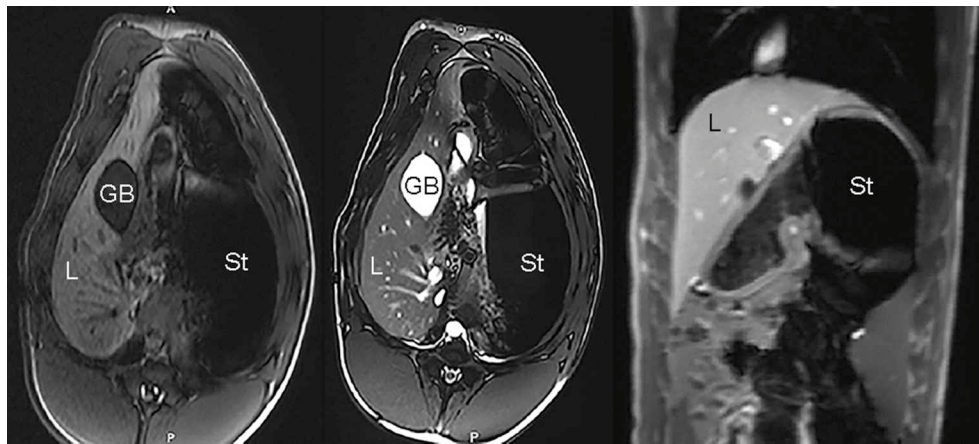


FIGURE 10 | Example of unremarkable porcine MR imaging study: axial T1-weighted image (**Left**), axial T2-weighted image (**Middle**), and coronal T1-weighted post-contrast image (**Right**); L, liver; GB, gallbladder; St, stomach.

financial burdens of clinical trials are—as previously noted—significant and time-consuming. Moreover, patient enrollment is challenging due to stringent eligibility criteria as well as competing clinical trials. Translational studies using validated animal models are thus critically essential in that they can efficiently and effectively undergo cohort clinical trial participation. This eliminates both accrual and logistical barriers to permit prospective early phase assessment of therapeutic modalities and to establish the validity of new technologies. Large animal models that faithfully recapitulate human patient tumor biology are particularly attractive for preclinical and co-clinical (parallel investigations in patients and animal cancer models to allow synchronization and real-time integration of preclinical and clinical efforts) trials for oncology. Given the integral role played by radiologic imaging in clinical trials, ensuring that medical imaging acquisition and interpretation is appropriately adapted to large animal cancer models is particularly important in developing the tools necessary for preclinical and co-clinical trial performance.

First, large animal imaging protocols and workflows must be optimized to ensure rapid performance and efficient interpretation of imaging. To this end, recent efforts have supported the development of clinically translatable porcine liver CT and MR imaging protocols using human clinical imaging systems. This has resulted in a customized and tested clinical imaging workflow (68). The developed CT (**Figure 9**) and MR imaging (**Figure 10**) protocols demonstrate consistent and reproducible, high-resolution radiologic depiction of the liver which parallels human patient imaging. The protocols support the capability to use advanced radiological imaging for diagnostic surveillance and therapeutic outcomes analysis. Second, the development and widespread utilization of centralized cloud-based radiologic picture archiving systems aimed at facilitating large animal imaging data capture and sharing is necessary to parallel digital imaging capture and centralized interpretation used in human clinical trials. Third, with limited published

experience reporting on large animal imaging and normal large animal radiologic anatomy, the range of normal findings must be defined through imaging of healthy subjects. Fourth, in the setting of disease, imaging findings for different large animal cancers must be validated against human cancer correlates, such that the specific imaging characteristics (location, morphology, vascularity, attenuation, signal, and avidity) of large animal disease parallel those seen in analogous clinical malignancies. Such validation may be pursued via systematic comparative radiology studies and radiologic-pathologic analyses. Fifth, the relative suitability of different imaging modalities for various large animal models, including dogs, primates, and pigs, requires exploration. Sixth, imaging benchmarks, diagnostic systems, and response assessment criteria need be extended to and standardized for all large animal platforms to allow investigators to make use of the range of available large animal models. To this end, current VCOG guidelines apply only to canine disease, though the use of pigs as a relevant large animal model is emerging (69). Such schemes must match validated systems which are recognized and employed by the clinical oncology community in order to enhance the relevance and applied translation to human clinical trials.

CONCLUSIONS

The emergence of numerous large animal oncologic models has provided a means for the study of cancer pathophysiology, and has allowed drug developers to systematically evaluate the effectiveness of new therapies and treatment strategies while avoiding some of the regulatory and financial burdens associated with conducting human clinical drug trials. These models provide an alternative to small animal models, which often do not adequately mirror the complex physiology seen in human tumor biology. With the increasing potential for prospective clinical trials using large animal models, care must be taken to create and adhere to standardized protocols, in order to ensure reproducible results and to allow for the accurate

comparison of study results across treatment strategies and sites. Clearly defined protocols for image acquisition and review are critical for the consistent handling of medical imaging data and objective assessment of response to therapy. Frameworks developed from human clinical trial image acquisition protocols, radiologic diagnostic schemes, and response assessment criteria can be tailored for use in large animal models, though care must be taken to ensure that such protocols are appropriately adapted to reflect nuances associated with specific models. Further validation of such animal models of disease and widespread adoption of universal protocols will help to

streamline the drug development process and improve the care of human disease.

AUTHOR CONTRIBUTIONS

FF and RG: substantial contributions to the conception or design of the work, acquisition, analysis, or interpretation of data for the work, drafting the work or revising it critically for important intellectual content, and provide approval for publication of the content. RG: guarantor of integrity.

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Porcine Models of Pancreatic Cancer

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Pancreatic cancer is the fourth most common cause of cancer-related deaths in both men and women. The 5-year survival rate for metastatic pancreatic cancer is only 8%. There remains a need for improved early diagnosis and therapy for pancreatic cancer. Murine models are the current standard for preclinical study of pancreatic cancer. However, mice may not accurately reflect human biology because of a variety of differences between the two species. Remarkably, only 5–8% of anti-cancer drugs that have emerged from preclinical studies and entered clinical studies have ultimately been approved for clinical use. The cause of this poor approval rate is multi-factorial, but may in part be due to use of murine models that have limited accuracy with respect to human disease. Murine models also have limited utility in the development of diagnostic or interventional technology that require a human-sized model. So, at present, there remains a need for improved animal models of pancreatic cancer. The rationale for a porcine model of pancreatic cancer is (i) to enable development of diagnostic/therapeutic devices for which murine models have limited utility; and (ii) to have a highly predictive preclinical model in which anti-cancer therapies can be tested and optimized prior to a clinical trial. Recently, pancreatic tumors were induced in transgenic Oncopigs and porcine pancreatic ductal cells were transformed that contain oncogenic KRAS and p53-null mutations. Both techniques to induce pancreatic tumors in pigs are undergoing further refinement and expansion. The Oncopig currently is commercially available, and it is conceivable that other porcine models of pancreatic cancer may be available for general use in the near future.

Keywords: pancreatic cancer, swine, porcine, transgenic, KRAS, p53

BACKGROUND: PANCREATIC CANCER

Pancreatic cancer (PC) is the twelfth most common cancer worldwide, with 460,000 new cases reported in 2018 (1). In the United States alone, it is estimated there will be 55,000 new cases of PC diagnosed in 2018, and 44,000 people will succumb to the disease (1). Over the last 40 years the demographic most affected by PC has been white men over the age of 60 (2). One of the main risk factors associated with development of PC is smoking, which is associated with a two-fold increase in incidence (2). Even with advances in our understanding of PC, the incidence has been rising ~0.5% each year over the last 10 years (2), and the 5-year survival rates in localized, regional (nodal spread), or metastatic disease have been 29, 11, and 2.6%, respectively (1–3). By 2030, PC is expected to be the second-leading cause of cancer mortality, which primarily is due to late presentation of symptoms and typically advanced disease stage at the time of diagnosis (2). Therefore, we need to improve our methods for diagnosing, detecting, and treating pancreatic cancer.

CURRENT AND EMERGING TREATMENT TRENDS FOR PC

The current treatment paradigm for PC involves surgery, radiotherapy, and chemotherapy (2, 4). Operative resection is still the preferred treatment for resectable tumors. Advancement in surgical and imaging technology likely contributed to a slight decrease in PC mortality in the early 2010's (2). In 1996, the first line treatment for patients with metastatic PC included gemcitabine (5). Combinational studies using gemcitabine with other agents failed to improve survival further until *nab*-paclitaxel was added (6, 7), which increased the median overall survival by 1.7 months compared to gemcitabine alone. However, this combination regimen has toxicity which excludes PC patients that have a poor performance status (6, 7). Another treatment option for PC is FOLFIRINOX (5-fluorouracil, irinotecan, and oxaliplatin), which resulted in a 4.3-month survival benefit compared to gemcitabine alone (8). These two treatment options, FOLFIRINOX and gem/*nab*-p, are the current best therapies until disease progression. Second-line treatment options include nanoliposomal irinotecan and 5-FU (approved in 2015), which improved median overall survival by 1.9 months compared to 5-FU alone (9).

Emerging treatment options for PC patients includes tumor microenvironment targeting (including immunotherapies), gene therapy, and PARP inhibitors. All immunotherapies are still in the clinical trial phase, with the most advanced trial involving CXCCesR4, a combination study with anti-CXCR4 (chemokine receptor) and anti-PD-1 (programmed cell death protein, an immune checkpoint inhibitor) (6, 10). In an open-label phase 1b study in patients that had disease progression while under treatment, combinatory therapy with a CC-chemokine receptor 2 (CCR2) kinase antagonist and FOLFIRINOX produced a tumor response in 49% of patients (6). A gene delivery system to deliver wild type p53 (SGT-53) into tumor cells is currently being tested in combination with gem/*nab*-p (6, 11). PARP inhibitors inactivate the repair mechanism for single-stranded DNA breaks (12, 13). These inhibitors induce cell death in tumors, and are given in combination with DNA-damaging agents. Clinical trials are currently underway for all of these emerging treatments for PC. For many of these novel therapeutic regimens, a highly-predictive preclinical model of PC might be helpful to assess and/or optimize the regimen prior to a clinical trial, which theoretically could reduce the risk of a failed clinical trial, thus decreasing (i) cost of drug development and (ii) strain on clinical resources. That is, a highly-predictive preclinical model of PC could streamline the drug development pipeline.

CURRENT ANIMAL MODELING OF PC

Similar to many human diseases, the study of PC has been aided by the use of genetically-edited murine models. Hallmark genetic mutations that drive the progression of PC have been well characterized (14–19). Oncogenic *KRAS* activation has been observed in 95% of PC patients, with 99% of point mutations occurring at the G12 position (20). Murine models have been

utilized to study *KRAS* and other genes involved with PC progression, including *TP53*, *SMAD4*, and *CDKN2A* (14, 18, 19, 21). Expression of the mutant *KRAS*^{G12D} in mice produced metastatic pancreatic tumors; duration of survival in these subjects decreased further with *TP53* antagonism (22). *TP53* is a well-known tumor suppressor that promotes apoptosis in response to cellular stress and DNA damage, and is mutated in 70% of PC patients (20). Furthermore, deletion of tumor suppressor genes (*SMAD4* or *CDKN2A*) enhanced tumor growth in a *KRAS*^{G12D} murine pancreatic cancer model (23, 24).

Despite the progress in genetically-edited murine PC models, a basic issue persists in regard to the mouse's relative ability to recapitulate human disease, including progression of PC and response to therapy. The magnitude of this issue is difficult to quantify using the current biomedical literature, in which many laboratories are heavily invested in the utilization of murine models. To be clear, it is not the intent of this article to criticize or discourage the use of mice in biomedical research, but rather to echo other voices which have questioned the predictive ability of murine models (25–27), and to propose alternative solutions. There has been some indirect evidence of murine fallibility in modeling human disease in the low regulatory approval rate for therapeutics that actually have reached the clinical trial stage, which has been in the range of 5–8% (28, 29). There are many factors that contribute to this low drug approval rate, but one likely reason is the less-than-optimal predictive ability of some murine models (e.g., tumor xenografting into immunosuppressed mice) to determine the efficacy of various therapeutics in humans (30–37).

Rodents may not accurately reflect human biology due to differences in physiology, anatomy, immune response, and genetic sequence (26, 30, 31, 36). For example, there are a number of genes for which the genotype-phenotype correlation is different between mice and humans (Table 1). One of these genes is *APC*^{+/-}, in which the human phenotype includes colorectal polyposis (leading to colorectal cancer); the murine *APC*^{+/-} mutant, however, develops small intestinal polyps. In addition, current genetically-edited murine models of cancer have limited tumor heterogeneity and low intratumor mutation rates (43–45), which could limit the clinical relevance of these models and their ability to study tumor immunity and immunotherapy (45, 46). And finally, there is a practical limitation to using murine models in preclinical research: size. Specifically, the development of clinically-relevant diagnostic or interventional technology often is not feasible with murine models due to their small size.

In fairness, murine models are being continually refined for cancer research, including genetically-engineered mouse models (GEMMs) as described above, mice with humanized immune systems (i.e., immunodeficient mice engrafted with human hematopoietic stem cells), and *in vivo* site-directed CRISPR/Cas9 gene-edited mice (25, 31, 47–49). Bacterial microbiota models also have been utilized to demonstrate the effects of bacteria on cancer development and progression in murine models; however the role of the microbiome has not yet been studied in large animal models of cancer (50). Though promising, these more sophisticated murine models come with increased

TABLE 1 | Comparison of phenotypes from the same genetic mutations between mice, pigs, and humans.

Mutated gene	Murine phenotype	Porcine phenotype	Human phenotype
<i>APC</i> (38)	Small intestine polyps	Colorectal polyps	Colorectal polyps
<i>CFTR</i> (39, 40)	Intestinal disease	Cystic fibrosis	Cystic fibrosis
<i>TP53</i> (41)	Axial skeleton tumors	Long bone tumors	Long bone tumors
<i>DMD</i> (42)	No phenotype	Progressive muscular dystrophy	Progressive muscular dystrophy

cost and complexity, and experience with them is still early. There remains a need for improved animal models of PC, including potential alternatives to mice, to better predict the human response to anti-cancer therapy. In addition, possession of an animal model of PC with human-sized organs would be helpful in regards to developing specific diagnostic and/or interventional technologies.

RATIONALE FOR A LARGE ANIMAL MODEL OF PC

As implied above, the rationale for utilizing a large animal model to study PC is to (i) have a platform for research and development of diagnostic/therapeutic technologies that would not be feasible in murine models, and (ii) to have a highly-predictive preclinical model in which emerging anti-cancer therapies could be vetted and optimized prior to clinical trial. Some current large animal models that are used for biomedical research include non-human primates, dogs, and pigs. Non-human primates are the most “human-like,” but there are societal and ethical concerns involved with the use of these animals for research (51, 52). Similarly, utilization of dogs in biomedical research also can bring up social concerns due to their role as companion animals (53). However, secondary to their relatively long life expectancy as companions, dogs have had some utility in the study of treatments for natural/inherent (i.e., age associated) tumors, including mammary carcinoma, prostate carcinoma, lymphoma, and various sarcomas (54).

Due to their size similarity with humans, various strains of pig have been used for years in biomedical research to develop and refine surgical equipment, instrumentation, and techniques (55). In addition, swine have greater similarity to humans with respect to genomic, epigenetic, physiological, metabolic, and immunological characteristics when compared to the mouse-human similarities (56–60). Generally speaking, the homology between the human and porcine genome is greater than the homology between the human and murine genome. A quantitative indicator of this genomic homology is difficult to generate and depends on the chosen endpoints, a discussion of which is beyond the scope of this review (55). However, these homologies have been estimated at 80–90% (human-porcine) and 60–70% (human-murine) (56, 61–63). Porcine models have been utilized to study a wide range of fields, including physiology, trauma, wound healing, and atherosclerosis (55, 59, 64). Along with primates, swine have been a favored model to study

transplantation (65). Human-pig concordance with regard to genotype-phenotype correlation is generally better than human-mouse concordance (Table 1). For example, the *CFTR*^{−/−} and *APC*^{+/−} mutants have the same basic phenotype in swine as in humans (38–40). Of note, a porcine genome map was generated in 2012, and further coverage, annotation, and confirmation is ongoing (60, 63, 66). Genetic manipulation of pigs (including knockouts, tissue-specific transgenics, inducible expression, and CRISPR editing), formerly done mostly in mice, has become more routine, with new gene-edited porcine models emerging for diseases such as atherosclerosis, cystic fibrosis, Duchenne muscular dystrophy, and ataxia telangiectasia (67–70).

Use of porcine models would offer other specific advantages. An animal research as large and robust as a pig would permit the testing of multiple, concurrent, clinically-relevant interventions, such as surgery, catheter-directed therapy, systemic chemotherapy, and/or radiotherapy; such combinatory interventions would have questionable feasibility in mice. Regarding the potential to study tumor biomarkers, the relatively large blood volume of a porcine PC model would allow for multiple blood samples to be drawn from the same pig during tumor development (a luxury not possible with the mouse), so precise timing and quantification of biomarker appearance could be correlated with tumor stage. This capability is not possible with a rodent model. On a similar note, immunotherapy study in a porcine PC model would be facilitated by the ability to obtain sufficient quantities of tumor-exposed immune cells that could be conditioned for re-infusion, e.g., as an autologous tumor-specific immunotherapy (71, 72). Furthermore, a porcine PC model could provide clinically-relevant tumor size/burden that would enable development and refinement of technologies to image and localize tumor for diagnosis, treatment, and surveillance (73). The relative size of the porcine subjects also would facilitate the sharing of tissue and blood sample with other investigators to a greater degree that could be accomplished with rodents. This effect would increase the potential number of investigators that could participate, the number of research protocols that could benefit, and the total amount of data that could be produced per research subject.

Of course, there are some caveats in using pigs to study PC. Specifically, the disadvantages of using a porcine model of PC with respect to a murine model include: (i) *Husbandry and Cost*. Depending on the swine strain utilized, the research subject could become quite large (>100 kg) if a prolonged (>1 year) latency is required for tumor development. Specialized equipment and experience would be necessary to handle such subjects.

Husbandry is generally more cumbersome and expensive with swine as compared to mice. (ii) *Biosafety*. Biosafety issues, particularly when working with recombinant DNA technology, become more complex when the subject is a pig that is housed in a pen, as opposed to a mouse inside a microisolator. (iii) *Aged Subject Availability*. While it is possible to work with aged murine subjects, and even elderly canine companion subjects, this is not really practical with swine, which potentially have a 20–30 year lifespan. Housing pigs for decades would be impractical, costly, and difficult, primarily due to the relatively large size of the mature subject (>150 kg for many strains). (iv) *Reagents and Tools*. Although use of swine in biomedical research has been growing, the availability of reagents and molecular tools specific for swine is not at the same level of availability that exists for mice. For example, the general availability of antibodies specific for porcine antigens is less than that for murine and human antigens. While difficult to quantify, in general this deficiency in porcine research is slowly improving. Of note, some anti-human antibodies will cross-react with porcine antibodies, but this has to be determined on a case-by-case basis. Secondary to these and/or other issues, it may not be practical or desirable for some research laboratories to utilize porcine models.

A TRANSGENIC APPROACH TO PORCINE PC MODELING: THE ONCOPIG CANCER MODEL

In 2012, the University of Illinois and the NSRRC (National Swine Resource and Research Center, nsrrc.missouri.edu) engineered a Cre-inducible swine model (the “Oncopig,” mini-pig background) (74) which carries an LSL-cassette containing dominant negative *TP53* (R167H mutation) and activated *KRAS* (G12D mutation); i.e., the porcine analog of the *KRAS*/p53 mouse (22). This Cre-inducible system allows for the expression of both mutations in any cell within the pig. Upon addition of adenovirus expressing Cre recombinase (AdCre) to cultured Oncopig fibroblasts, expression of both mutant *KRAS* and *TP53* was noted (74). The transformed fibroblasts had a shorter cell cycle length and demonstrated *in vitro* “tumorigenic” properties (increased cell migration, soft agar colony formation) and formation of tumors when injected into immunocompromised mice (74). Injection of AdCre into the subcutaneous/intramuscular regions of the Oncopig resulted in tumor formation with pleomorphic features (74). This transgenic pig hence became known as the Oncopig Cancer Model (OCM).

Primary pancreatic ductal cells were cultured from the OCM and then infected with AdCre; these epithelial cells also displayed a transformed phenotype *in vitro*, and expressed mutant *KRAS* and *TP53* (75). These transformed epithelial cells were injected into SCID mice and formed subcutaneous tumors that were histologically and phenotypically similar to human pancreatic ductal adenocarcinoma (PDAC) (75). *In vivo* injection of AdCre directly into the main pancreatic duct of an Oncopig resulted in several nodular tumors after 12 months. Comparison of tumor induced in the OCM pancreas with human PDAC revealed similar morphological features, including a dense desmoplastic

stromal reaction that is one key hallmark features of human PDAC (75). In addition, increased expression of proliferative markers (ERK and PCNA) was present in the OCM pancreatic tumor (75).

Key features of modeling PC with the OCM include: (1) the initial tumor induction is genetically defined; (2) the induced tumor is autochthonous; (3) the host has an intact immune system, which is capable of producing an anti-tumor immune response similar to humans, for studying immunotherapies (76); and (4) the tumor induction procedure (AdCre injection) is relatively simple and safe. However, there are some potential issues, such as specificity. Injection of AdCre theoretically could result in non-specific infection of multiple cell types, producing a pleomorphic tumor which could detract from the clinical relevance of the model. There also may be an issue of tumor latency with pancreatic tumor in the OCM; in the initial report (75), pancreatic tumor formation required 12 months, and this was not visible on computed tomography nor was it clinically apparent. So, further refinement of the OCM for PC studies might be beneficial.

ORTHOTOPIC APPROACH: TRANSFORMED PORCINE PDECs

In contrast to the autochthonous mechanism of tumor induction that the OCM provides, an orthotopic method of tumor induction involves seeding of tumorigenic cells into the pancreas, preferably into an immunocompetent host. In pursuit of this model type, primary cultures of porcine pancreatic ductal epithelial cells (PDECs) were established from explants of normal pancreatic tissue; IHC for cytokeratin-19 in early-passage strains were consistent with epithelial origin of the cultured cells (77). Strains of PDECs subsequently were infected with a lentiviral vector containing GFP, *TP53*^{R167H}, and *KRAS*^{G12D} (LV-GKP; generated using porcine sequences), producing clones with demonstrable expression of mutant p53 and *KRAS*; refer to **Table 2** (77). Initial *in vitro* tumorigenic assays of these clones (denoted as PGKP, for PDECs transformed with LV-GKP) demonstrated increases in migration and soft agar colony formation relative to primary PDECs (77). To further increase the transformed phenotype of the PGKP cells, RNAi of SMAD4 and CDKN2A were added using additional LV vectors, with ~70–90% knockdown (77). Relative to primary cells, these secondary clones (PKGPs and PGKPSC) also displayed increased proliferation, soft agar colony formation, invasion, and migration, i.e., evidence of *in vitro* “tumorigenicity” (77), with perhaps enhanced capabilities compared to the primary clone (PGKP cells). The three types of transformed PDECs (summarized in **Table 2**) were then implanted subcutaneously in nude mice; all three cell lines formed tumors and demonstrated equivalent *in vivo* tumorigenicity (77). In summary, PDEC-derived tumorigenic cell lines were established, which currently are undergoing orthotopic implantation into syngeneic, immunocompetent domestic swine.

In terms of generating pancreatic tumor, the theoretical advantages of transformed PDEC implantation over AdCre

TABLE 2 | Characteristics of transformed porcine ductal epithelial cells [data published as preprint (77)].

	KRAS ^{G12D}	p53 ^{R167H}	SMAD4 shRNA	p16 ^{Ink4A} shRNA	Colony formation	Proliferation	Migration	Invasion	Xenografts
PGKP	+	+			+++				+
PGKPS	+	+	+		++	+++	+	++	+
PGKPSC	+	+	+	+	+++	+++	+	++	+

P, porcine epithelial cells; G, GFP; K, KRAS^{G12D}; P, p53^{R167H}; S, SMAD4; C, CDKN2A/p16. Transformed phenotypes of porcine pancreatic ductal epithelial cells in vitro and in vivo. Scale of transformation +++ > ++ > +.

injection in the OCM include: (i) *Specificity*. the former technique only involves transformed pancreatic ductal cells, meaning that tumor induced with transformed PDEC implantation would be more likely to originate from a specific cell type than tumor induced with AdCre injection in the OCM. (ii) *Target Flexibility*. Cell implantation permits the investigator to choose the targets by which transformation will be accomplished, instead of being restricted to mutant KRAS and TP53, as in the OCM. (iii) *Host Flexibility*. The investigator can choose the background strain of pig (or another species altogether) with cell implantation, while the OCM by definition involves one transgenic genotype. (iv) *Cost*. The purchase price of OCM subjects likely will be greater compared to most strains of research-quality pigs (though this cost differential becomes less of an issue in the face of multiple months of housing that these experiments would require).

On the other hand, the potential disadvantages of transformed PDEC implantation with respect to AdCre injection in the OCM include: (i) *Immune Rejection*. If allogeneic transformed PDECs are implanted, then there is the possibility that the host would reject the transplanted material (this issue might be minimized by utilizing syngeneic or autologous PDECs). (ii) *Simplicity*. AdCre injection into the OCM is straightforward and has potentially fewer Biosafety issues, as compared to pancreatic harvest, primary cell culture, and numerous viral transformations required for the PDEC implantation technique. (iii) *Local Environment*. As discussed above, tumor induction in the OCM is autochthonous, and likely does not involve local traumatic disruption of tissue architecture which presumably ensues when a cellular suspension is injected. However, the amount and biological relevance of local architecture disruption in these models is not known at this time.

APPLICATIONS AND IMPACT

The availability of a validated, genetically-defined porcine model of PC would have multiple potential applications, including (in no particular order):

1. Development and refinement of catheter-based technologies for diagnosis and/or intervention.
2. Discovery and study of serum tumor biomarkers ("liquid biopsy" technology).
3. A preclinical trial tool: a penultimate platform to test novel chemotherapeutic agents that were screened in murine models, prior to pushing a nascent therapy into an expensive clinical trial.

4. A platform for the testing of multiple, concurrent, clinically-relevant interventions, such as surgery, catheter-directed therapy, systemic chemotherapy, and/or radiotherapy (as described under the Rationale section).
5. Study of early events in tumor initiation and progression in an animal subject with a relatively high degree of genetic, physiological, metabolic, immune, and anatomic similarity with humans.
6. Detailed study of tumor heterogeneity (facilitated by a relatively large tumor specimen).
7. Study of the interactions and effects of the microbiome on tumor biology.
8. Development and refinement of tumor-visualization aids (such as fluorescent tumor agents) to assist with R0 resection in surgery.
9. Development and refinement of tools for open and minimally invasive surgery.
10. Refinement of existing imaging tools (such as MRI-based technologies) to diagnosis early stage tumors.
11. Development of novel tumor imaging tools.
12. An educational tool to instruct trainees in surgical resection techniques.

The primary impact of such a porcine PC model would be to increase the efficiency and safety at which impactful technologies and therapies could be brought into the clinical realm. For example, the anti-tumor effect and toxicity of a new chemotherapeutic regimen could be vetted in the porcine model, which could promote (or eliminate) the regimen's introduction into a clinical trial; this screening step likely would increase the probability of success for the human study. As another example, the feasibility, safety, and utility of a catheter-directed energy source in the treatment of PC could be accomplished in a porcine model without ever having to place a patient at risk. Another impact of a porcine model of PC would be an increased understanding of the molecular and cellular biology of the disease in an animal model that would have more relevance than the mouse.

CONCLUSION AND FUTURE DIRECTIONS

Current murine models of PC have been tremendously helpful in the progression of understanding and treatment for this disease, but there is an ongoing issue of the relative predictive ability of these murine models. The issue of modeling accuracy likely has contributed in part to an unacceptably high failure rate of experimental therapeutics in clinical trials.

Utilizing pigs to model PC has potential benefits, including relevant subject size, increased genetic homology, and better immunological/metabolic mimicry with respect to humans. Specifically, the size of pigs allows for improvement upon imaging and surgical techniques which is not possible with rodents. The OCM has already demonstrated that pancreatic tumor can be induced in the pig with histopathological features similar to human PC. This PDAC model will provide ways for improving early detection, imaging, and surgical techniques of PDAC by following the disease after a defined induction point. Even though the current OCM does have some limitations due to the amount of time it takes to develop tumors, this model potentially could be refined to accelerate tumor growth; for example, by introducing additional edits within the Cre-recombined cells that would inhibit DNA repair and promote genomic instability, or by generating a tissue-specific inducible promoter for targeted initiation of cellular transformation upon AdCre administration. Another approach to generate a porcine PC model has been orthotopic implantation of transformed PDECs into the pancreas of the syngeneic, immunocompetent pigs. Additional approaches to pancreatic tumor induction in the pig might include direct pancreatic infection with viral vectors containing key tumor-associated

gene sequences, *in vivo* CRISPR editing, or combinations of two or more of the technologies described herein. To address the issue of tumor induction in relatively young subjects, diet-induced metabolic syndrome could be used as an adjunctive measure, which likely would increase the physiological age of the subject (and mimic a common clinical co-morbidity). Work remains to be done in the development and validation of a tractable porcine model of PC. Once established, however, a porcine PC model should be a useful addition to the armamentarium of the PC researcher, and should be able to augment and/or complement work done with established murine models.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Cellular Therapies for the Treatment of Hematological Malignancies; Swine Are an Ideal Preclinical Model

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The absence of clinically relevant large animal tumor models has historically forced experimental cellular therapies for hematological malignancies to translate directly from murine models to clinical trials. However, recent advances highlight swine as an ideal large animal model to demonstrate the safety of murine proof of concept studies prior to their implementation clinically. The availability of the MHC defined MGH miniature swine herd has been key for the development of novel approaches for hematopoietic cell and solid organ transplantation. New spontaneously arising hematological malignancies in these swine, specifically myeloid leukemias and B cell lymphomas, resemble human malignancies, which has allowed for development of immortalized tumor cell lines and has implications for the development of a large animal transplantable tumor model. The novel development of a SCID swine model has further advanced the field of large animal cancer models, allowing for engraftment of human tumor cells in a large animal model. Here, we will highlight the advantages of the swine pre-clinical model for the study of hematological malignancies. Further, we will discuss our experience utilizing spontaneously arising tumors in MGH swine to create a transplantable tumor model, describe the potential of the immunodeficient swine model, and highlight several novel cellular and biological therapies for the treatment of hematological malignancies in swine as a large animal pre-clinical bridge.

Keywords: miniature swine, lymphoma and leukemia, transplantation, cell therapy, SCID

INTRODUCTION

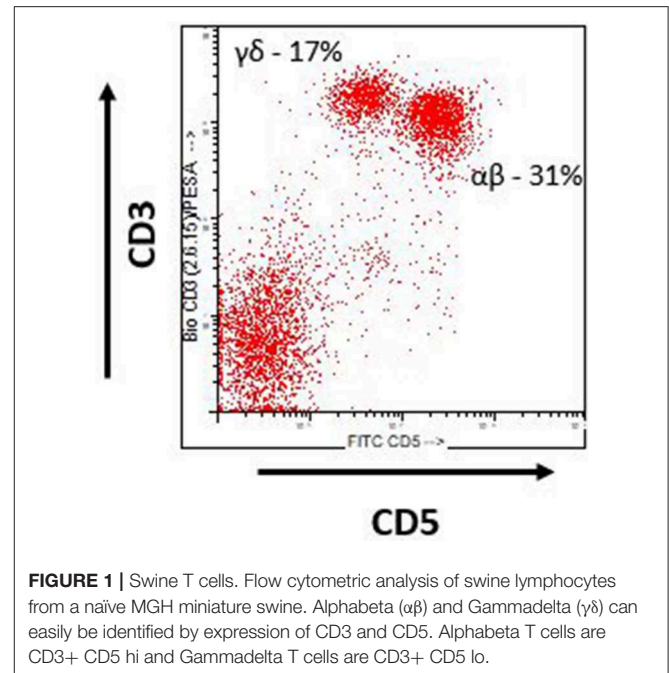
Preclinical murine models have long been the foundation for mechanistic studies and assessment of therapeutic strategies for human disease. While the mouse has provided a cheap, reproducible, and easy to use model whose role will never be replaced, the extrapolation of mouse studies directly to clinical application has largely been unsuccessful, especially with respect to cancer (1–3). This is likely due to the vast number of genetic, immunologic, and physiological differences between mice and humans. Murine models often recapitulate a specific pathway within a disease, but frequently do not provide the entire spectrum of physiologic changes that occur in humans, preventing direct translation of therapeutic strategies. Large animals provide a more clinically relevant model to study cancer as they are significantly more similar to humans in terms of anatomy, physiology, genetics, and immunological responses. Some however, may challenge the ethical aspects of using large animals for research purposes. Among the large animals used for pre-clinical research purposes, primates, canines, and swine are the three most common. Primates are most similar to humans

with respect to physical and anatomic characteristics (4, 5), and there are an abundance of human reagents with cross-reactivity to primates. However, the use of primates in research is often hindered by strict regulations, potential for communicable diseases, the requirement for significant personnel training and personal protective equipment, societal protest, and expense. Canine and swine models provide a more practical option with respect to ease of breeding and handling, shorter gestation periods, and large litters, while maintaining an anatomy and physiology that is similar to humans (6). To date, there are a limited number of large animal models of hematological malignancies (7–10). However, the existing models, specifically swine models, have demonstrated that large animal hematological malignancies share important similarities to human malignancies (11–13). Further, the swine model is increasingly being used in the setting of anti-cancer drug development (14). Here we will highlight the advantages of the swine pre-clinical model for the study of hematological malignancies, while also reviewing existing swine models and exploring novel therapeutic strategies, both existing and on the horizon (8, 15).

SWINE AS A PRECLINICAL MODEL OF MALIGNANCY

Swine as a preclinical model of hematologic malignancy offer several advantages over other species one of which is a similar immune profile, specifically the lymphocyte repertoire. Despite the similarities, there are several important differences to note. With respect to T cell populations, both humans and swine possess two distinct lineages of T cells based on the alphabeta or gammadelta T cell receptor (16). Alphabeta T cells in both species recognize foreign antigen in an MHC dependent fashion, while gammadelta T cells recognize foreign antigen in a non-MHC dependent fashion. One major difference however is the fact that swine possess significantly higher numbers of gammadelta T cells than do humans, particularly in the peripheral blood and intestinal lymphoid tissues (16). Experimentally, swine alphabeta and gammadelta T cells can be easily distinguished based on CD3 and CD5 expression utilizing flow cytometry (Figure 1). As a result, swine represent an ideal model for the study of gammadelta T cell responses in the setting of malignancy, a previously underexplored area.

Both swine and humans possess traditional T helper cells, CD4+CD8⁻, which recognize antigen in a MHC class II dependent manner, and cytolytic T cells, CD4⁻CD8⁺ which recognize antigen in a MHC class I dependent manner. Swine however possess significantly more cytolytic CD8⁺ T cells than T helper CD4⁺ cells in circulation, which is the opposite of the human T cell repertoire (16). Further, while CD4+CD8⁺ T cells exclusively reside in the thymus in humans, CD4+CD8⁺ T cells can be found in extrathymic locations in swine, and are differentiated from CD4+CD8⁺ thymocytes by expression of CD8 alpha alpha homodimer and lack CD1 expression. Peripheral CD4+CD8^{lo} cells are memory T helper (Th) cells, distinct from naïve Th cells which are CD4+CD8⁻. These



memory Th cells acquire the CD8 alpha alpha homodimers as a result of antigen exposure (16).

Another advantage of the swine model is the abundance of swine specific reagents available. Historically, a barrier in swine research has been the relative lack of swine specific reagents. However, a recent study outlined enormous progress on this front, specifically identifying swine cluster of differentiation (CD) markers and linking them to their human counterparts (17). A broad literature review identified 359 known swine CD markers, with over 800 identified reagents including monoclonal antibodies, polyclonal antibodies, and fusion proteins against 266 swine CD markers. With respect to *in vitro* monitoring of the immune system (e.g., flow cytometry), there are commercially available porcine antibodies directed against every major cell type including porcine T cells, B cells, NK cells, T regulatory cells, myeloid cells, dendritic cells, neutrophils, and others. *In vivo*, there are a host of swine specific reagents as previously mentioned including depleting antibodies targeted against CD3, CD4, CD8, and Tregs (17). The effects of a novel rabbit anti-porcine anti-thymocyte globulin (ATG) have been investigated. Rabbit anti-human ATG is a commonly used agent clinically in the setting of conditioning prior to hematopoietic cell transplantation (HCT), treatment of graft vs. host disease (GVHD), and for treatment of acute cellular rejection after solid organ transplantation. In swine, rabbit anti-porcine ATG is a poor T cell depletion agent (unpublished data). In comparison, two anti-CD3 immunotoxins were superior. The chemically conjugated swine anti-CD3-immunotoxin provides robust T cell depletion in swine (18) while a recombinant (less toxic version) was also relatively effective with a 80% decrease in CD3 T cells in the peripheral blood (19). These findings have been previously documented in other species and humans supporting that monoclonal antibodies are less potent at immunodepletion

within tissues when compared to immunotoxins (20–22). There have also been several porcine recombinant fusion toxins generated, specifically a porcine IL-2 fusion toxin for *in vivo* depletion of swine CD25+ cells and a porcine CTLA-4 fusion toxin for depletion of antigen presenting cells (APCs) (23). There is also evidence that human therapeutics can cross react with corresponding porcine targets *in vivo* with great efficacy (24).

Finally, given the recent shift in treatment of cancer toward immunotherapies, there is a growing need for new biomarkers that are predictive for treatment stratification, monitoring and response. Swine are an ideal model for the discovery and validation of novel biomarkers given their physiologic and immune similarities to humans as described previously. Advantages include the ability to longitudinally follow swine over a period of years given their long life span and the relative ease in obtaining large quantities of blood, serum, and tissue samples (25). Importantly, existing swine models of cancer have demonstrated similarities in biomarkers compared to their human counterparts. In an oncopig model of hepatocellular carcinoma (HCC), alpha feto protein (AFP) was reliably used for detection of swine HCC as well as treatment monitoring (26). With respect to hemolymphatic malignancies, in swine PTLT, LDH is a reliable marker of hemolysis and tumor development (13). The development of reliable swine models of hemolymphatic malignancies has enormous potential to uncover novel biomarkers.

LARGE ANIMAL MODELS OF LYMPHOHEMATOPOIETIC MALIGNANCIES

We previously reported our identification of spontaneously developing chronic myelogenous leukemia (CML) in the Massachusetts General Hospital (MGH) major histocompatibility complex (MHC) defined miniature swine herd (11). Through years of selective breeding, the MHC genes of these swine have been “fixed,” while minor antigens remain variable, thereby providing a valuable large animal model to study transplantation. The CML that spontaneously develops in these swine (sCML) closely resembles human CML (hCML) as confirmed by flow cytometric analysis of peripheral blood mononuclear cells (PBMCs), lymph nodes (LNs), as well as histological examination of tissues obtained at necropsy (11) (**Figure 2**). The development of hCML is closely associated with a chromosomal translocation $t(9;22)$, also known as the Philadelphia chromosome (Ph+) in over 95% of cases. sCML cell lines isolated from MGH miniature swine were karyotyped to evaluate for an analogous chromosomal translocation. Although the direct translation of a $t(9;22)$ translocation could not be made due to disparities in chromosome numbers (23 pairs in humans vs. 19 pairs in swine), a shortened chromosome arm was found, indicating that the development of sCML is likely associated with a chromosomal abnormality. Interestingly, sCML was associated with defects in a nucleoporin gene

(Nup107). Defects in this gene have also been associated with human leukemias (27, 28).

The identification of a severe combined immunodeficiency (SCID) pig at Iowa State University has offered a potentially valuable model for the study of hematological malignancies (29). These naturally occurring SCID pigs were found to have two causative mutations in the Artemis gene, a well-characterized gene in human SCID patients (30). SCID pigs share a similar immune profile to that of human SCID patients as they are completely deficient in T and B cells and are thus incapable of producing antibodies or mounting T cell responses. Similar to humans, the SCID pig does have macrophages and natural killer (NK) cells, the latter of which are primarily responsible for the immune response in these animals. The success of engraftment of human hematopoietic stem cells in xenotransplantation studies in mice relies, in part, on the ability of polymorphisms within the murine non-obese diabetic (NOD) signal regulatory protein alpha (SIRPA) gene that dictate its capability to be activated by human CD47 (31) on hematopoietic cells. Signaling of these two molecules confers phagocytic tolerance to human stem cells by the murine monocytic/macrophage innate immune arm. Interestingly, macrophages from SCID pigs did not reject human lymphohematopoietic cells, thus demonstrating a NOD phenotype (32). However, xenogeneic tumor studies in which human pancreatic and melanoma cell lines were introduced into SCID pigs revealed an NK cell infiltrate in tumors in a subset of pigs. Despite this NK cell infiltrate, these pigs did not reject the xenografts (33). The authors hypothesized that the lack of rejection in the setting of an NK cell response was secondary to a deficiency in cytokine production, namely IL-2. Finally, Powell et al. recently reported the development of a spontaneous host-derived T cell lymphoma and a chronic lymphocytic leukemia (CLL) following bone marrow transplantation (BMT) in two SCID pigs (34). The development of a host-derived malignancy following BMT may be related to a “leaky” Artemis gene that allows for generation of lymphocytes, albeit at reduced numbers, as previously documented in human SCID patients (35). Moving forward, whether malignancies arise spontaneously or are introduced from allogeneic or xenogeneic origins, it is clear that the SCID pig will become a potent tool for studying lymphohematopoietic malignancies. Moreover, as the first large animal model to allow for engraftment of human cancer cell lines without concern for rejection, the SCID pig will be invaluable for testing of novel cellular and pharmacological therapies.

Previously we reported the use of the MGH miniature swine as a potential model of B cell lymphomas, specifically post-transplant lymphoproliferative disease (PTLD), which is a potentially lethal complication following transplantation (12, 36). In our experience with both hematopoietic cell transplantation (HCT) and solid organ transplantation (SOT), MGH miniature swine develop PTLT as a result of uncontrolled herpes viruses, either from primary infection or reactivation of a gammaherpesvirus, porcine lymphotropic herpesvirus-1 (PLHV-1) (36) (**Figures 3A,B**). Clinically, herpes induced lymphomas (HILs) are observed in immunosuppressed patients, such as those with HIV or transplant patients. However, in humans, PTLT is driven by primary infection or reactivation

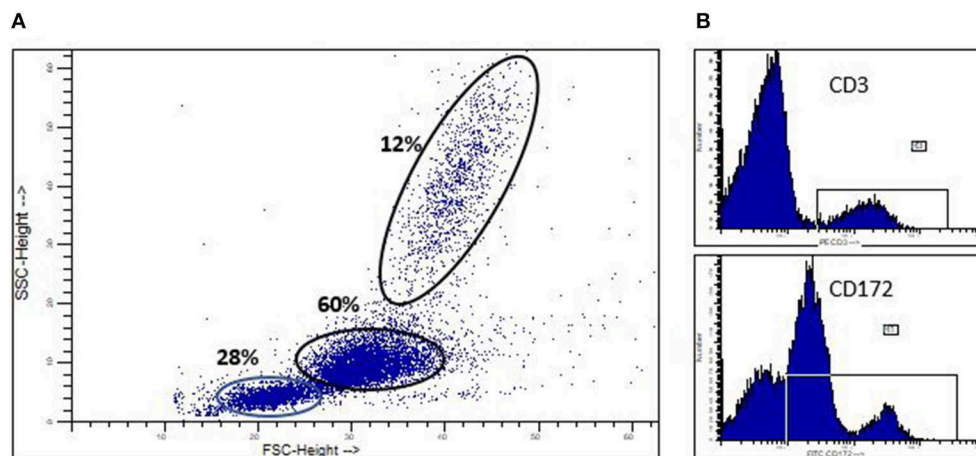


FIGURE 2 | Spontaneously arising swine CML. **(A)** Flow cytometric analysis of peripheral blood in a swine which spontaneously developed CML, demonstrating a significant myeloblastosis. **(B)** CD3+ and CD172+ staining demonstrating the malignancy is of myeloid origin (CD172+) and not lymphocyte origin (CD3+).

of Epstein Barr virus (EBV) (13) as a result of loss of antiviral function of CD8+ cytotoxic T cells in the setting of immunosuppression. Unfortunately, there is currently no animal model that accurately recapitulates EBV-induced PTLD. To date, rodent models continue to be the most utilized when studying PTLD, with novel therapies being tested in murine xenogeneic models using human PTLDs (37). However, these rodent models cannot accurately replicate potential complications due to their small size (38). Other studies using murine gammaherpesvirus have struggled with their inaccuracy modeling human disease (38). In future studies, the SCID pig may provide an exciting model in which to study EBV driven PTLD in an animal of human size and physiology. It is important to mention that the model is not devoid of limitations. Besides the restrictions of working across xenogeneic barriers, the inherent fragility of SCID swine [which are highly susceptible to infections (35)] and the requirement of housing with room-sized specialized (Biobubbles) and hepa-filtered (ABSL-3 like) animal facilities may prove difficult to many due to expense. However, for the first time, this model will allow for the assessment of novel human derived cellular therapies and pharmacological approaches to address PTLD in a large animal model.

SWINE AS A PRE-CLINICAL MODEL TO TEST BIOLOGICAL AND CELLULAR THERAPIES

Cellular therapies such as blood transfusions have been used in medicine for decades. One of the most sophisticated cellular therapies—bone marrow transplantation (BMT)—has evolved dramatically since its inception in 1956 and is now used clinically to treat a variety of hematological malignancies and blood dyscrasias (39). During BMT, the recipient's immune system is (partly or fully) destroyed by radiation or chemotherapy and replaced by either autologous or allogeneic bone marrow. Unfortunately, frequent complications

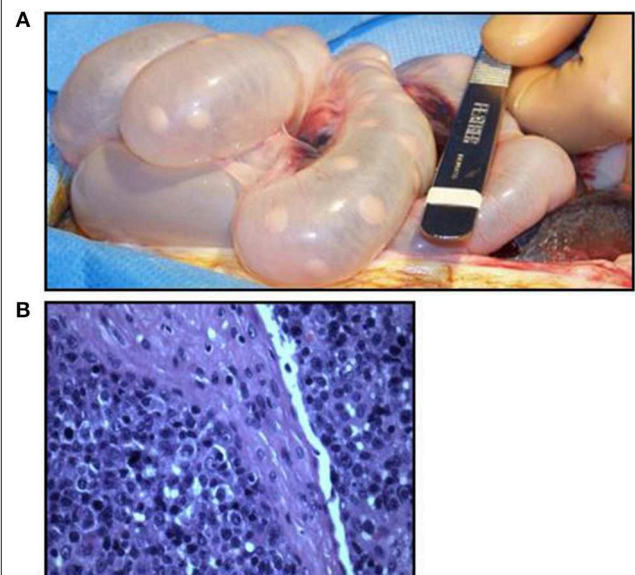


FIGURE 3 | Swine PTLD. **(A)** Significant intestinal lymphadenopathy in a swine with PTLD. **(B)** Histologic analysis of a swine intestinal lymph node demonstrating an acute lymphoblastic process in the setting of PTLD.

of allogeneic bone marrow transplantation are infection and graft-vs.-host disease (GvHD), during which donor immune cells attack recipient cells. Swine (40–42), as well as other animal models (43, 44), have played an important role in the study and development of BMT and other cellular therapies for clinical application. Swine are an attractive model for the study and development of cellular immunotherapies due to the abundance of swine-specific reagents available (45–52). We have previously demonstrated the applicability of swine as a clinically-relevant model for the use of cellular therapies in inducing immunological tolerance via mixed chimerism (in which both recipient and

donor cells co-exist) as well as for the treatment of GvHD in the form of donor leukocyte infusions (DLIs) (42, 53). In the following, we will recap some of these studies and touch on novel cellular therapies on the horizon.

Donor Leukocyte Infusions (DLI)

The use of donor leukocyte infusions (DLI) following allogeneic HCT to both augment anti-tumor responses and enhance immune cell engraftment has expanded dramatically since its introduction 30 years ago (54). DLIs utilize donor peripheral blood leukocytes collected via apheresis, a process in which blood components are separated via density gradient and lymphocytes and monocytes are harvested, while granulocytes are returned to the patient. DLI following HCT in the setting of hematological malignancy has several indications, including as a prophylactic therapy for patients with a high risk of relapse, treatment of PTLD and viral infections and as a rescue therapy for those with graft failure. However, GVHD remains one of the most feared side effects of allogeneic BMT. Important studies by Sachs' group demonstrated the value of swine as a cell therapy model to optimize and harness the anti-leukemia effects of allogeneic HCT while avoiding GvHD. These studies in swine exploited a novel HCT approach first shown in mice in which the establishment of mixed chimerism across MHC barriers promoted immune tolerance, thus preventing GvHD (55, 56). This study and other similar studies in non-human primates laid the foundation for Sachs' pioneering study in humans using allogeneic BMT to simultaneously treat multiple myeloma and induce immune tolerance to MHC mismatched kidney allografts (57). Using the swine model described above, we attempted to leverage the alloreactive properties of DLIs to enhance donor chimerism, thereby maintaining or preventing graft loss, while at the same time avoiding GvHD (42). In our study of a total of 33 clinically dosed DLIs infused to immune tolerant swine chimeras, 21 failed to induce conversion to full donor hematopoietic chimerism or cause GvHD, demonstrating that our reduced intensity conditioning regimen for HCT promotes mixed chimerism and an immune tolerant state that is strongly resistant to DLI and GvHD. In several animals, we were able to demonstrate that DLI mediated GvH reactivity could be overcome by significantly increasing the DLI dose, removing chimeric host peripheral blood cell populations (thought to be regulatory T cells) through extensive leukapheresis of the recipient immediately prior to DLI, or delivering lymphocytes fully mismatched to host MHC, but not to donor MHC. However, conversion to full donor chimerism in these scenarios was often associated with severe GvHD, highlighting the importance of mixed chimerism for maintaining immune tolerance. More refined DLIs with selected effector populations are currently being developed and swine will play an important role in determining their efficacy.

$\gamma\delta$ T Cells Infusions

$\gamma\delta$ T cells are a conserved subset of T cells with a distinct surface receptor and which mediate innate immune responses and promote immune surveillance (58). The abundance of $\gamma\delta$ T cells in humans ranges from 1 to 20% in the peripheral blood and constitutes the major cell population in skin and mucosa

(59). Swine $\gamma\delta$ T cells have been previously characterized and can be tracked in the peripheral blood and tissues using the monoclonal antibody PPT27 (58, 59). $\gamma\delta$ T cells play a known role in the anti-tumor response and can therefore potentially be used as a potent cellular therapeutic in the context of BMT. However, infusion of donor type $\gamma\delta$ T cells in a murine model of acute GVHD (aGVHD) mice increased the severity of aGVHD while the absence of host type $\gamma\delta$ T cells was associated with reduced antigen presenting cell (APC) activation and aGVHD in an MHC-mismatched model (59). By contrast, aGVHD severity was not altered in a MHC-matched, minor antigen (miHA) disparate model of HCT (58, 60). Studying the role of $\gamma\delta$ T cells in the setting of MHC disparity using a large animal model may prove useful amidst growing evidence of the immune modulatory effects of these cells.

$\gamma\delta$ T cells provide potent anti-tumor responses to both solid and hematopoietic malignancies, including lymphoma and multiple myeloma (61). As opposed to $\alpha\beta$ T cells, $\gamma\delta$ T cells are not MHC restricted when it comes to antigen recognition and do not require APCs for processing immunogenic peptides, allowing them to quickly reactivate during an immune response. Interestingly $\gamma\delta$ T cells can be activated and expanded *in vivo* through the use of bisphosphonates, which inhibit farnesyl pyrophosphate synthase (62). Taken together, the unique immune properties of $\gamma\delta$ T cells, as well as their ability to be activated *in vivo* using conventional drugs, make them an attractive option for experimental use in swine models of HCT. Thus far, there have been limited studies utilizing $\gamma\delta$ T cells in preclinical models or clinical settings. Using a large animal preclinical model such as swine, where $\gamma\delta$ T cells have been well-characterized, could allow for optimization of important parameters including dosing, route (systemic vs. intratumoral), kinetics, and *ex-vivo* manipulation. More importantly, safety studies in an outbred swine will help discern the conflicting $\gamma\delta$ T cell GVHD murine studies and facilitate the expanded use of $\gamma\delta$ T cells in clinical settings.

NK Cell Therapies

NK cells are a type of innate lymphoid cell that mount allogeneic immune responses in a non-MHC restricted manner. NK cells distinguish "self" vs. "non-self" through interactions between the killer inhibitor receptor (KIR) expressed on their surface and HLA class I expressed on the surface of host tissues. Recognition of "self" HLA class I by the KIR results in an inhibitory signal, while the absence of HLA class I expression stimulates NK cell activation. NK cells play a pivotal role in the anti-tumor response in cancer cells that down-regulate HLA expression to escape recognition by T cells.

Clinically, NK cells provide a powerful anti-leukemia effect in the setting of allogeneic HCT. For the treatment of acute myelogenous leukemia (AML), donor NK cell alloreactivity from KIR mismatched donors displayed an anti-leukemia effect as part of T cell depleted grafts, while simultaneously providing protection against GVHD (63). Based on these anti-leukemic effects in the absence of GVHD, adoptive NK cell therapy has also been studied as an alternative to unmanipulated DLI for leukemic relapse. NK-DLI was demonstrated to be both a feasible and

safe option in a study of 30 patients receiving nonmyeloblastic allogeneic stem cell transfer (SCT). CD56⁺ selected NK cells were given as an NK-DLI 8 weeks after initial transplant. Patients tolerated the DLI well without significant GVHD (64). In the non-transplant setting, administration of KIR mismatched NK cells in 10 pediatric patients with AML who had achieved complete remission following chemotherapy resulted in transient engraftment and excellent two year overall survival (100%) (65). Studies are also underway to evaluate the potential for activated NK cell therapy in the setting of refractory lymphoma (66).

The recent development of the SCID pig provides an interesting avenue for studying the role of NK cells in both allogeneic and xenogeneic anti-tumor responses in a large animal model (67). Similarly to human NK cells, swine NK cells can be identified by expression of CD3⁺CD16⁺CD56⁺ surface markers (68). Powell et al. demonstrated that the number of NK cells in SCID pigs is approximately eight times higher than in non-SCID pigs and that these NK cells are intrinsically functional, as demonstrated by their ability to be activated *in vitro* and lyse tumor cells at the same rate as NK cells from non-SCID pigs (69). In the absence of circulating T and B cells, methods to activate and harness NK cell immune responses can be specifically evaluated in SCID pigs. For example, as IL-2 can stimulate NK cells against ovarian cancer in a murine model (70), further evaluation of IL-2 and other therapies in the SCID pig may further delineate the clinical relevance of NK cells in anti-leukemia and anti-lymphoma immune responses.

Chimeric Antigen Receptor (CAR) Cells

CARs are genetically engineered receptors that reprogram T cells to target specific cell surface antigens without the need for MHC interaction. CAR T cell therapy has revolutionized cancer research through the re-direction of T cells to target surface receptors expressed by tumor cells, most notably CD19 which is expressed on B cell malignancies (71). CAR T cells were first shown to have anti-tumor responses in mice and then in humans with refractory hematological malignancies (71–73). Currently, groups are designing novel CARs for applications other than cancer, such as autoimmunity and infectious disease (74). As a result, there is an increased need to test the safety of many of these. Indeed, fatal side-effects have been observed in clinical trials, which argues for the need for improved safety testing prior to clinical application, ideally in large animal models (75).

Given the availability of swine models of B cell lymphomas and myeloid leukemias and the identification of the SCID pig, swine may provide an ideal large animal model for the testing of CAR therapies. Though further refinement of these models is necessary, their use should be encouraged for safety assessments in pre-clinical studies. Because CARs rely on the insertion of gene sequences coding for a monoclonal antibody (with a given antigen specificity) as part of the receptor, a potential limitation of swine models is that the CAR may not recognize the target antigen on swine cells. Though this is a potential limitation, for some constructs with conserved antigens, CARs can be very informative for “off-target effects” (76). Swine may be particularly valuable for assessing the relative kinetics and persistence of

individual CARs, as it was recently shown that 4-1BB CARs are longer lived when compared to CD28 CARs in humans (77). The potential use of SCID pigs engrafted with human leukemias/lymphomas to assess cytotoxicity/clearance, dosing, imaging, CAR surveillance and different systemic/local delivery methods may be revolutionary in a field that has been mostly limited to murine models. Furthermore, swine could also be used to test the efficacy of anti-PTLD therapies by either CARs directed to PLHV-1 in the MGH swine or to EBV CARs in the case of humanized swine. The possibilities are endless depending on the approach and research questions asked, and highlight the potential role of swine as a critical player in the pre-clinical space of cellular immunotherapies.

Immune Checkpoint Blockade

Activation of the host immune system against invading tumor cells has long been the goal of cancer therapeutics. A major breakthrough in this endeavor was the discovery of immune checkpoint proteins, which serve to downregulate the immune response. The first immune checkpoint protein to be well-characterized was cytotoxic T-lymphocyte-associated protein 4 (CTLA4), a receptor found on the surface of regulatory T cells and activated T cells. When CTLA4 is bound to its ligands, CD80 and CD86, on the surface of APCs, it provides an inhibitory signal to the T cell. However, the activation molecule CD28 also binds to CD80/CD86, albeit with a reduced affinity as compared to CTLA4. Thus, in the setting of solid organ transplantation where the goal is to suppress the host immune response to the allogeneic graft, it was hypothesized that CTLA-4 would block CD28 interactions with CD80/86, thereby preventing T cell activation. In support, Belatacept, a novel CTLA4 Ig fusion protein, is an effective form of immunosuppression in organ transplantation in both large animal models and humans (78, 79). Naturally, in the setting of cancer, blocking the interaction between CTLA4 and CD80/86 would serve to activate circulating T cells and theoretically fight off invading tumor cells. Ipilimumab, a monoclonal antibody directed against CTLA-4, was first approved by the FDA in 2011 for the treatment of metastatic melanoma and has provided excellent results (80).

The porcine version of CTLA-4 (pCTLA4) exists in several forms and can suppress human CD4⁺ T cell responses costimulated by porcine B7. Utilizing a novel diphtheria toxin (DT) based recombinant pCTLA4 fusion toxin, Peraino et al. demonstrated effective binding to CD80 expressing porcine cells and subsequent inhibition of protein synthesis in those cells. Follow up studies in mice inoculated with a CD80⁺ porcine lymphoma cell line showed that mice injected with the DT based pCTLA4 fusion toxin experienced prolonged survival compared to untreated mice. It remains to be seen whether the use of pCTLA-IT has similar effects in swine as what has been observed in murine studies where blocking or removing (genetically) host APCs diminished GVHD by limiting the direct activation of alloreactive T cells (81). In summary, the demonstrated ability of a DT based pCTLA4 to inhibit growth of porcine lymphoma cells provides a foundation for future work in targeting CTLA4 in large animal models of lymphohematopoietic malignancies.

CONCLUSIONS

Cancer research is currently being revolutionized by the development of novel cellular and genetic therapies. Historically, these strategies required testing in small animals due to the absence of reliable large animal cancer models. However, recent advancements in swine including the development of immortalized myeloid and lymphoma cell lines from inbred MHC characterized swine, the accessibility of genetically engineered oncogenic swine (known as onco-pigs and addressed in a companion review in this series), and the ability of engrafting human tumors in the SCID

pig, highlight swine as an ideal model for large animal tumor studies.

AUTHOR CONTRIBUTIONS

RD-S: Lead the topic. CH: Provided figures and edited the paper with modifications. RD-S and AM: Wrote the manuscript.

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Translating Human Cancer Sequences Into Personalized Porcine Cancer Models

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The global incidence of cancer is rapidly rising, and despite an improved understanding of cancer molecular biology, immune landscapes, and advancements in cytotoxic, biologic, and immunologic anti-cancer therapeutics, cancer remains a leading cause of death worldwide. Cancer is caused by the accumulation of a series of gene mutations called driver mutations that confer selective growth advantages to tumor cells. As cancer therapies move toward personalized medicine, predictive modeling of the role driver mutations play in tumorigenesis and therapeutic susceptibility will become essential. The development of next-generation sequencing technology has made the evaluation of mutated genes possible in clinical practice, allowing for identification of driver mutations underlying cancer development in individual patients. This, combined with recent advances in gene editing technologies such as CRISPR-Cas9 enables development of personalized tumor models for prediction of treatment responses for mutational profiles observed clinically. Pigs represent an ideal animal model for development of personalized tumor models due to their similar size, anatomy, physiology, metabolism, immunity, and genetics compared to humans. Such models would support new initiatives in precision medicine, provide approaches to create disease site tumor models with designated spatial and temporal clinical outcomes, and create standardized tumor models analogous to human tumors to enable therapeutic studies. In this review, we discuss the process of utilizing genomic sequencing approaches, gene editing technologies, and transgenic porcine cancer models to develop clinically relevant, personalized large animal cancer models for use in co-clinical trials, ultimately improving treatment stratification and translation of novel therapeutic approaches to clinical practice.

Keywords: personalized cancer models, exome sequencing, gene editing, translational research, clinical needs

INTRODUCTION

The global incidence of cancer is rapidly rising, and despite an improved understanding of cancer molecular biology, immune landscapes, and advancements in cytotoxic, biologic, and immunologic anti-cancer therapeutics, cancer remains a leading cause of death worldwide. The 14.1 million new cancer cases diagnosed in 2012 are expected to dramatically increase over the next decade to 19.3 million annual cases by 2025 (1). Cancer is caused by the accumulation of a series of gene mutations

called driver mutations that confer selective growth advantages to tumor cells (2). The development of next-generation sequencing technology has made the evaluation of mutated genes possible in clinical practice, allowing for identification of driver mutations underlying cancer development in individual patients. This, combined with frequency and function-based methods allows for distinguishing potential driver mutations from passenger mutations that have no effect on tumorigenesis. These advances have provided unique insights into the wide variety of genetic alterations present in an individual patient's tumor, and have spurred interest in utilizing this information to inform treatment stratification. However, translation of this genomic information into improved therapeutic approaches has not been successful for the majority of cancer patients. Therefore, as cancer therapies move toward personalized medicine, improved modeling capabilities for predicting the role driver mutations play in therapeutic susceptibility are required to address this unmet clinical need.

Recent advances in gene editing technologies such as CRISPR-Cas9 have enabled development of tumor models with specific genetic driver mutations. When applied to murine cancer models, these targeted genetic alterations have provided key insights into key mutational events promoting tumor progression and altered response to therapy (3, 4). However, many drugs showing promise in murine studies fail to translate into successful clinical trials (5), highlighting the need for improved models to better translate therapeutic efficacy, optimal dosing, and ideal combination therapies to clinical practice. Pigs represent an ideal animal model for development of genetically defined tumor models due to their similar anatomy, physiology, metabolism, immunology, genetics, and epigenetics compared to humans (6–14). In addition, their similar size permits utilization of the same instrumentation and technical maneuvers used in humans and optimized by clinicians, facilitating rapid clinical translation.

As cancer therapies move toward personalized medicine, predictive modeling of the role driver mutations play in tumorigenesis and therapeutic susceptibility will be essential. Combining porcine cancer models and gene editing technology would allow for development of clinically relevant personalized tumor models for prediction of treatment responses for mutational profiles observed clinically. Such models would support new initiatives in precision medicine, provide approaches to create disease site tumor models with designated spatial and temporal clinical outcomes, and create standardized tumor models analogous to human tumors to enable therapeutic studies. In this review, we discuss the process of utilizing genomic sequencing approaches, gene editing technologies, and transgenic porcine cancer models to develop clinically relevant, personalized large animal cancer models for use in co-clinical trials, ultimately improving treatment stratification, and translation of novel therapeutic approaches to clinical practice.

INFLUENCE OF DRIVER MUTATIONS ON TREATMENT RESPONSE

Cancer is caused by the accumulation of a series of gene mutations called driver mutations that confer selective growth

advantages to tumor cells (2). The development of next-generation sequencing technology has made it possible to evaluate mutated genes in tumor cells. This, combined with frequency and function-based methods allows for distinguishing potential driver mutations from passenger mutations that have no effect on tumorigenesis. Although our understanding of the role various mutations play in driving tumorigenesis is incomplete, it is clear that genetic mutations are found in all cancers, some of which have been associated with biological characteristics of cancer (15). While all tumors result from genetic mutations, each tumor type develops mutations at different rates. In the instance of HCC, it is estimated that a single tumor contains 30–40 mutations on average, 5–8 of which are likely driver mutations (16, 17). Some of these driver mutations can have profound effects on tumor biology, having significant implications regarding diagnostics, prognostics, and therapeutic responses. For example, mutation of the tumor suppressor gene *TP53* is associated with poor prognosis and doxorubicin resistance in HCC (2, 18–20), while RAS activation is associated with resistance to sorafenib (2). Other examples include *KRAS* mutations associated with epidermal growth factor receptor antibody resistance in colorectal cancer (15), and *BRAF*^{V600E} mutations associated with positive response to vemurafenib in melanoma patients (21). As genomic analyses of clinical cancer samples continues to increase, and databases such as The Cancer Genome Atlas (TCGA) continue to grow, so does our understanding of the mutations that impact treatment recommendations. However, despite the knowledge that driver mutational profiles can have significant impacts on treatment responses, tumor genomic information is not routinely used when considering treatment strategies for the vast majority of cancer types. The lack of translation into actionable therapeutic modalities highlights the need to develop novel platforms to rapidly analyze and predict therapeutic responses for patients based on their driver mutational profiles.

CO-CLINICAL TRIAL CONCEPT

With increased interest in testing targeted therapeutics based on driver mutational profiles in cancer patients comes a significant decrease in the number of relevant patients available for enrolment in appropriate clinical trials, significantly reducing the number of new targeted and combination therapies that can be tested. One of the new ways investigators are attempting to address this issue is through the use of co-clinical trials. Co-clinical trials are defined by the National Cancer Institute (NCI) as parallel or sequential trials of combination therapy in patients and in mouse and human-in-mouse models of appropriate genotypes to represent the patients. Utilization of mouse models that mimic the genetics of human disease in parallel to early phase human clinical trials can assist in treatment stratification by identifying patient populations most likely to benefit from treatments based on their genetic makeup. These so called “mouse hospitals” enable testing of drugs in murine models representative of multiple cancer subtypes while minimizing the cost, time, and number of human patients required (4). Co-clinical trial approaches using genetically engineered mouse models (GEMMs) have shown promise for

screening therapeutics and identifying patient populations that would benefit from specific treatments (4). However, GEMMs have several drawbacks that limit the translatability of results to clinical practice. The metabolic rate of mice is substantially higher than in humans (22), and vast differences in drug metabolism and xenobiotic receptors make rodents poor models of toxicity, sensitivity, and efficacy when used in preclinical drug studies (23). The ability to establish toxicity and drug sensitivity in animal models is immensely important, as <8% of cancer drugs translate successfully from animal model testing into Phase I clinical trials (24). In addition, their small size prohibits the utilization and testing of the same tools and techniques employed in clinical practice. This is particularly important given the recent expansion of targeted locoregional ablative and arterial therapeutic strategies that reduce systemic toxicities and increase tumor drug delivery. This, combined with the fact that the genetic events required for mouse tumorigenesis differs from humans (25), highlights the need for development of improved animal models to facilitate translation of targeted and personalized therapeutic strategies to clinical practice.

Argument for Porcine Cancer Models

Given the limitations of currently available murine and other small animal cancer models, there is a pressing need to incorporate large animal cancer models into preclinical and co-clinical therapeutic testing approaches. Pigs represent an ideal platform for development of genetically defined large animal cancer models due to their similarities with humans in size, anatomy, physiology, metabolism, genetics, epigenetics, and immunology (6–14). The life cycle of pigs also allows for development, characterization, treatment, and follow-up in a clinically relevant timeframe (26). The availability of many outbred porcine lines, high homology between the pig and human genome (27, 28), and conservation of epigenetic regulatory patterns (13) highlights the relevance of genetically defined porcine cancer models and their ability to mimic the genetic variation observed in patient populations. Pigs are also ideal models for investigation of chemotherapeutic toxicity, as the animal's basal metabolic rate and xenosensor pregnane X receptor—which is responsible for the metabolism of half of all prescriptions drugs (29)—are also very similar to humans (30, 31). Finally, their similar size allows for utilization of the same tools and techniques used in clinical practice. This is particularly important for cancers where systemic chemotherapeutic administration offers only marginal survival benefit with poor quality of life, as procedural approaches using locoregional therapeutic approaches are potentially curative therapeutic options that require further preclinical testing, but cannot be tested using similar tools in smaller animal models.

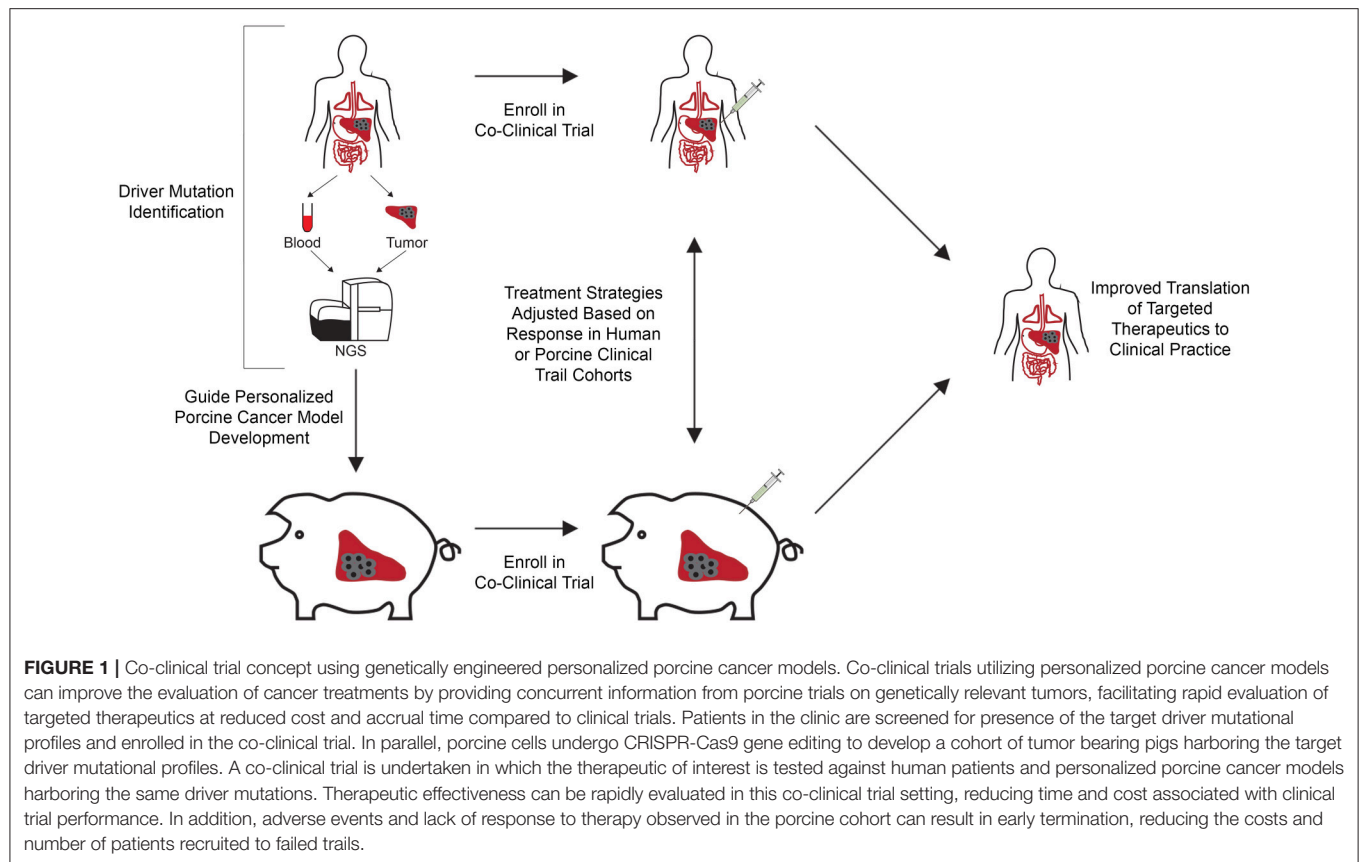
Until recently the only porcine cancer models available were spontaneous or chemically induced models (32–34). However, the sequencing of the pig genome in combination with the recent advances in targeted genome editing approaches such as CRISPR-Cas9 has allowed for development of genetically defined porcine cancer models. To date a number of genetically defined porcine cancer models capable of mimicking histological and transcriptional hallmarks of human cancer, as well as

responses to cancer drug therapies have been developed. These include the Oncopig Cancer Model—a transgenic pig model that recapitulates human cancer through induced expression of heterozygous *KRAS*^{G12D} and *TP53*^{R167H} driver mutations—which has been utilized to develop HCC (35), pancreatic cancer (36), and soft-tissue sarcomas (37, 38), and a heterozygous *TP53* knockout model of spontaneous osteosarcomas (39). As genetically defined porcine cancer models continue to be developed, their use in co-clinical trial formats could provide improved prediction of patient populations that would benefit from specific treatments, improving translation of novel, targeted, and combination therapeutic strategies from preclinical murine studies to clinical practice. As our understanding of driver mutational profiles commonly observed in clinical practice continues to expand thanks to the increased use of genomic sequencing in clinical research, this information can serve as a basis for generation of additional porcine cancer models using CRISPR and somatic cell nuclear transfer (SCNT) technologies.

While there are a number of benefits associated with the use of genetically defined porcine cancer models in co-clinical trial settings, these models are not without limitation. Drawbacks of using porcine models as opposed to murine models include increased housing and husbandry requirements due to their increased size and lifespan. This limitations also limits the ability to develop, breed, and distribute multiple strains of porcine cancer models harboring different driver mutations as is currently done for murine models. In addition, specialized equipment and experience are required to ensure safe and ethical handling and use of pigs for testing experimental treatments. These animals are also raised in controlled environments that do not mimic the environmental conditions human patients are exposed to—although as this limitation is shared with murine and other cancer models, a detailed discussion of the environmental factors impacting tumor biology and treatment response is outside the scope of this review. Finally, the costs associated with development, maintenance, and utilization of porcine cancer models in co-clinical trials is significantly higher than murine models, although their use would come at a reduced cost compared to those associated with human clinical trial participants.

TREATMENT STRATIFICATION UTILIZING PERSONALIZED PORCINE CANCER MODELS

Our increased understanding of the unique genetic makeup of each patient's tumor has shed light on the fact that individual cancer varieties exist, and therefore therapies need to be optimized and adjusted to effectively treat individual patients. This optimization requires the use of preclinical cancer models representative of the driver mutational profiles of individual patients. While current co-clinical trials seek to utilize genetically defined murine cancer models in combination with human cancer patients to evaluate treatment response for patient populations, personalized porcine cancer models could transform precision medicine by providing a means to



significantly improve the predictability of safety and efficacy of therapeutic drugs, devices, and procedures in co-clinical trial settings (**Figure 1**). Below we outline the process for developing genetically defined, personalized porcine cancer models, using the Oncopig HCC model as an example.

The first step in developing personalized porcine tumor models consists of identification of the driver mutational profile for which the treatment in question is most likely to be effective against. For targeted therapeutics, this can be done using preclinical murine models prior to proceeding with co-clinical trials utilizing personalized porcine tumor models. For repurposed compounds already approved for other cancer types, this would require knowledge of the driver mutational profiles of responding and non-responding patients. This requires performance of biopsy collection, followed by DNA extraction and genomic sequencing—for example through whole genome or whole-exome sequencing—to identify the driver mutations present. Sequencing of a control sample, such as blood, is also required to assist in distinguishing between germline and somatic mutations. Utilizing genome editing approaches such as CRISPR-Cas9, driver mutations associated with improved outcomes can be introduced into the porcine HCC cells *in vitro*. Following screening to identify cells containing the desired driver mutational profile, HCC cells are propagated for autologous injection, resulting in development of pigs bearing HCC tumors with driver mutational profiles representative of the patients

of interest. Utilizing this approach, a cohort of personalized porcine cancer models can be developed in a timely fashion and utilized in co-clinical trials, significantly reducing the costs and accrual time associated with clinical trials. This approach would also provide significant benefits over murine co-clinical trials by utilizing a model animal with similar metabolism and size to humans, allowing for the same tools and techniques to be employed in both human and porcine subjects.

While the above example describes utilization of the Oncopig Cancer Model to develop personalized HCC tumors, this approach is not limited to the Oncopig and can be adjusted to facilitate development of personalized tumors for a wide variety of cancer types. However, due to the above mentioned challenges associated with developing, breeding, and disseminating multiple strains of porcine cancer models, it is unlikely that the breadth of porcine cancer models required for co-clinical trials targeting specific driver mutational profiles will ever match the number of commercially available murine models. Therefore, development of various cohorts of genetically defined porcine cancer models for co-clinical trials will likely depend on utilization of CRISPR-Cas9 to induce tumors harboring desired driver mutational profiles in individual wild type or previously produced inducible porcine cancer models. While this approach provides additional challenges compared to utilization of genetically defined murine models, it also allows for rapid development of genetically defined porcine tumor models without the extended time

required to develop a pig herd harboring the desired mutational profile. In this regard, pigs harboring tumors representative of multiple driver mutational profiles could be used as their own control to confirm the effects of a given driver mutational profile on treatment response. This approach could also revolutionize personalized medicine by facilitating development of genetically unique, patient specific tumors for performance of therapeutic trials on tumors representative of the genetic profile of individual patient tumors. However, much work is still required to make this approach feasible in a timely and cost efficient manner.

Accounting for Intratumoral Heterogeneity

One of the challenges faced when developing personalized tumor models is accounting for intratumor heterogeneity, which describes the accumulation of different genetic mutations in tumor cells within a single tumor as tumor cells evolve (40). Knowledge of the genetically diverse cell populations within a tumor can be important for guiding optimal cancer treatment decisions, and therefore the effectiveness of personalized tumor models to predict the optimal treatment strategy may be underappreciated when used to treat heterogeneous tumors. While tumor cells representative of the driver mutations modeled will be killed, the patient may develop a recurrent tumor or not respond at all due to proliferation of resistant tumor cells. These situations highlight the importance and significant challenge associated with performing clinical and co-clinical trials for targeted therapeutics, as well as the challenges of successfully employing them in clinical practice. While modeling heterogeneity represents a significant challenge for animal cancer models, the need to perform gene editing on individual pigs as described above provides an avenue through which tumor heterogeneity can be accounted for using personalized porcine cancer models. Porcine cell lines representative of multiple driver mutational profiles can be developed, mixed, and injected to develop *in vivo* intratumor heterogeneity representative of the patient population of interest. In this case, therapies will only prove effective if they're capable of eradicating all of the

genetically distinct tumor cells present. Another option would consist of development of individual tumors representative of one of the genetically diverse tumor cell populations. Using this approach, treatment strategies can be applied to tumors representative of different driver mutational profiles in isolation, allowing for identification of treatment strategies most effective for each tumor cell population. However, these approaches do not take into account additional challenges associated with modeling tumor heterogeneity, including accurate identification of individual tumor clones, effects of cellular signaling and interactions between tumor cells with differential mutational profiles, and the impact of germline mutations on tumor biology.

CONCLUSIONS

Advances in sequencing and gene editing technologies have provided significant insights into the impact of driver mutations on treatment responses for a wide range of cancer types; however, translation of this genomic information into improved therapeutic approaches has not been successful for the majority of cancer patients. We present a new personalized porcine cancer model approach leveraging clinical genomic sequence information, gene editing technologies, and transgenic porcine cancer models to develop clinically relevant, personalized large animal cancer models to better predict response to treatment in co-clinical trial settings, ultimately improving treatment stratification and translation of novel therapeutic approaches to clinical practice. Furthermore, as these techniques continue to improve, this approach could revolutionize personalized medicine by facilitating development of genetically defined porcine cancer models representative of individual patients for performance of personalized therapeutic trials.

AUTHOR CONTRIBUTIONS

CX, SW, LS, and KS conceptualized and wrote the manuscript.

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Exploring the Potential Utility of Pet Dogs With Cancer for Studying Radiation-Induced Immunogenic Cell Death Strategies

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Radiotherapy serves as a foundational pillar for the therapeutic management of diverse solid tumors through the generation of lethal DNA damage and induction of cell death. While the direct cytotoxic effects of radiation therapy remain a cornerstone for cancer management, in the era of immuno-oncology there is renewed and focused interest in exploiting the indirect bystander activities of radiation, termed abscopal effects. In radioimmunobiologic terms, abscopal effects describe the radiotherapy-induced regression of cancerous lesions distant from the primary site of radiation delivery and rely upon the induction of immunogenic cell death and consequent systemic anticancer immune activation. Despite the promise of radiation therapy for awakening potent anticancer immune responses, the purposeful harnessing of abscopal effects with radiotherapy remain clinically elusive. In part, failure to fully leverage and clinically implement the promise of radiation-induced abscopal effects stems from limitations associated with existing conventional tumor models which inadequately recapitulate the complexity of malignant transformation and the dynamic nature of tumor immune surveillance. To supplement this existing gap in modeling systems, pet dogs diagnosed with solid tumors including melanoma and osteosarcoma, which are both metastatic and immunogenic in nature, could potentially serve as unique resources for exploring the fundamental underpinnings required for maximizing radiation-induced abscopal effects. Given the spontaneous course of cancer development in the context of operative immune mechanisms, pet dogs treated with radiotherapy for metastatic solid tumors might be leveraged as valuable model systems for realizing the science and best clinical practices necessary to generate potent abscopal effects with anti-metastatic immune activities.

Keywords: immuno-oncology, comparative oncology, abscopal, immunogenic cell death, radiation, canine, metastases

SIGNIFICANCE OF A DOG MODEL—STRENGTHS AND LIMITATIONS

Strengths

Domesticated dogs are second only to human beings in terms of being afflicted with naturally-occurring and inherited diseases, and the purposeful breeding of companion dogs for specific characteristics has produced lineage-specific homogeneity that mimics human demographics such as race or geographic phenotypes (1–4). Dogs acquire genetic diseases as do humans, and consequently might serve as suitable comparative models for conserved pathologies, including certain types of cancer (5, 6). Given that pet dogs often share the same environment and are exposed to similar carcinogens as people, the natural development and evolution of canine tumors can closely parallel those that afflict human beings and share comparable recurrence and metastases patterns. The compressed lifespans of dogs in comparison with humans, combined with the substantial veterinary healthcare dollars spent on pet dogs exceeding \$15 billion annually (7), provide researchers with a robust population of pet dogs available to participate in studies of cancer pathogenesis and the preclinical assessment of investigational therapeutics and medical devices (8–11). Collectively, the shared genetics of specific canine cancers with their human counterparts (12–17), and the high societal value placed upon dogs as companion animals, uniquely and ethically allow pet dogs to serve as potential valuable large animal models for translational cancer research. Particularly, in the era of immuno-oncology, pet dogs might uniquely serve as ideal parallel tumor models, given the development of spontaneous cancers under competent immune surveillance mechanisms which invariably contributes to shaping of cancer cell immunogenicity and the associated immune topography of the tumor microenvironment (18, 19).

Limitations

While the recognition of comparative oncologic pathology has been existent for over 50 years (20), the establishment of comparative oncology as a health science discipline by the National Cancer Institute's Center for Cancer Research remains relatively nascent, being formalized in 2003. As such, the purposeful inclusion of pet dogs as parallel cancer models for investigational anticancer immunotherapeutic strategies has only recently begun to bear scientific results in support of the potential model value (21), and has not been maximally leveraged by the scientific cancer research committee given the existence of perceived and true barriers (9), which include heterogeneity of study populations and tumor biology, necessity to conduct adequately powered and prospective clinical trials, and limited availability of diagnostic and therapeutic tools for in-depth scientific investigations. For the study of anticancer immune responses, the diversity and number of commercially available and validated reagents for characterizing immune activation in the domestic canine remain limited in comparison to the existent murine and human reagent toolboxes (22, 23). Additionally, the nuances of immune composition and activation responses in canines is less well-annotated compared to traditional

inbred mouse strains (24–26), however, in aggregate there is sufficient data to support the comparative similarities for specific aspect of the immune system between canines and humans (27, 28).

To expedite the translation of novel immune-based strategies to people with metastatic tumor histologies, the evaluation of experimental therapies in the most highly relevant tumor models should be considered. Besides people, domesticated dogs are also large mammals that develop solid tumors spontaneously that are not only metastatic, but also immunogenic and include canine oral malignant melanoma (OMM) and appendicular osteosarcoma (OS) (29, 30). Importantly, studies demonstrate that these 2 specific solid tumors share similar genetic and histologic features as those found in humans (31–35); suggesting that pet dogs might serve as excellent predictive models for guiding the rational development of immune-based strategies in people with comparable tumor histologies (36).

IONIZING RADIATION THERAPY

Radiation Principles and Mechanisms of Cell Death

The biologic responses of cells exposed to radiation traditionally have been categorized into the 5 R's, being Repopulation, Reassortment, Reoxygenation, Repair, and Radiosensitivity. Understandings of these foundational cellular reactions to ionizing radiation have been leveraged to maximize the anticancer activities of radiation therapy (37, 38). The primary target for radiation cellular damage is DNA, and with low linear energy transfer radiation, such as photons and electrons, single strand DNA breaks are created, accumulate, and mimic damage similar to double strand breaks that become difficult, if not impossible, to repair. Consequently, irreparably damaged cells can no longer replicate limitlessly, and the primary cause of cellular death is mitotic catastrophe (39, 40). Irradiated cells can also undergo apoptosis rapidly following radiation exposure with this form of death most relevant to lymphoid cells (39). Other death pathways also play roles in response to radiation, including autophagy and necrosis. Autophagy involves internal degradation of organelles for the promotion of cellular survival and occurs after radiation as a survival mechanism; but can also progress to cellular death and influence inherent radiosensitivity (41, 42). Lastly, by extensive cellular stress through DNA damage, radiation can induce cellular senescence with consequent tumor cell growth arrest (43, 44).

Radiation-Induced Immunogenic Cell Death and Abscopal Effects

While anticancer activities from radiation have traditionally been ascribed to direct DNA damage to tumor cells, in the era of immuno-oncology, there has been focused interest to understand the indirect or “out-of-field” immunomodulatory activities induced by radiation therapy. Specifically, a unique form of radiation-induced cell killing called immunogenic cell death (ICD) holds promise for activating systemic immunity against tumor masses distant from the field of radiation

delivery (45), a phenomena termed abscopal effect (46). The regressive activity of local irradiation on distant metastatic cells, constituting the abscopal effect, is attributed to an immune-mediated response (47). Given the recognized potential to amplify systemic anticancer immunogenicity following localized radiation, excitement has been garnered by the scientific community to understand and harness the promise of radioimmunotherapy (48, 49).

Mechanistically, ICD has been a focus of radiobiology research and requires activation of the innate immune system through the release of damage-associated molecular patterns (DAMPs) or alarmins, which are released from injured, stressed, or dying cells within the radiation field (50). Scores of different endogenous alarmins derived from cellular organelles and extracellular matrix proteins have been described (51); however, three specific molecules appear to be required for optimal dendritic cell activation and immune priming against malignant cells, specifically being membrane localization of calreticulin and the release of high mobility box group 1 (HMBG1) and adenosine triphosphate into the tumor microenvironment (52). Collectively the expression and secretion of alarmins by dying cells create a localized milieu which exert either “eat me” or “come find me” signals, and are capable of activating innate immune cells exhibiting cognate DAMPs receptors (TLR, RAGE, P2X7), which leads to the priming of cytotoxic T lymphocytes for an adaptive anticancer immune response (53). Given their immune activating properties, the purposeful induction of alarmins within the tumor microenvironment as an *in-situ* vaccine strategy is actively being investigated (54, 55).

While the elicitation of ICD within the primary tumor microenvironment through ionizing radiation has potential to prime the innate immune system, there remains the necessity for generating sufficient out-of-target tumor responses known as the abscopal effect, especially at sites of metastatic burden that might be unamendable to conventional localized treatment strategies. Despite the documentation of abscopal activities induced by localized radiation therapy in combination with adjunctive treatments (cytokines and chemotherapy), the fraction of human cancer patients that reliably demonstrate abscopal activities sufficient to induce macroscopic tumor regression remains <30% (56). The contextual scenarios (tumor type, host environment, therapeutic combinatorial sequencing) by which abscopal effects can be generated by radiation therapy remain incompletely defined (57, 58). As such, prospective investigations with high-value animal models could accelerate the identification of ideal circumstances to augment the proportion of human cancer patients whom might benefit from the life-extending activities of radiation-induced ICD and associated abscopal effects.

Opportunity to Optimize Radiation-Induced ICD Protocols

While several recent investigations have discussed the optimal dose and timing of radiation therapy relative to immunologic intervention, no single protocol is clearly superior to others, and the impact of dose rate is relatively unexplored. Given the

non-uniformity of various therapeutic radiation regimens for the management of diverse solid tumor histologies, a significant research barrier exists for the thorough characterization of contributory radiation variables required for optimal radiation-induced ICD. While recent meta-analysis has been conducted to “standardize” immune activating potential of radiation treatment protocols through the comparison of biologic effective dose in preclinical models (59), there remains a scientific need for additional prospectively-designed studies inclusive of model systems that more faithfully recapitulate the natural progression of cancer development under immune evolutionary pressures. This “gap” in knowledge given the absence of an ideal experimental model system, is underscored by the rarity of achieving radiation-induced abscopal effects in human cancer patients (56, 60–62). As such, the consistent and reproducible generation of clinically meaningful abscopal effects in most cancer patients remains infrequent and suggests that the current state of understanding regarding radiation-induced immune activation remains incomplete and necessitates the inclusion of complementary innovative modeling systems.

One mechanism to generate new knowledge regarding the feasibility and limitations of radiation-induced ICD and associated abscopal effects could include the rational inclusion of pet dogs with solid tumors. Therapeutic management of cancer in pet dogs parallel the same modalities in human cancer patients, with the inclusion of radiation therapy for controlling localized tumor progression and associated morbidity. Importantly, the repertoire of cognate receptors including toll-like receptors responsible for detecting the presence of pathogens (pathogen associate molecular patterns) and danger signals (damage associated molecular patterns) have been recently characterized in the domestic canine (26, 63–65). With existing tools and knowledge of radiobiology and immunology in the canine species, an opportunity exists to prospectively and systemically evaluate novel radiation-induced ICD strategies in pet dogs that could be translated into life-extending abscopal activities in human cancer patients.

RELEVANT SOLID TUMORS IN PET DOGS FOR OPTIMIZING RADIATION ABSCOPAL EFFECTS

Canine Oral Malignant Melanoma (OMM)

Malignant melanoma is a metastatic solid tumor affecting both dogs and people (66), however, the anatomic locations of primary tumors differ, with oral cavity and skin being the primary sites for malignant melanoma in dogs and humans, respectively. In canines, melanoma is considered the most common oral malignancy, accounting for ~40% of all oral cancers (67). Despite differences in primary anatomic site, prominent molecular drivers of malignancy are conserved between dogs and people, including AKT and MAPK signaling pathways (31).

Effective management of canine OMM requires local treatment strategies, as well as systemic intervention to delay the onset and progression of regional and/or distant metastases

TABLE 1 | Summary of canine melanoma immunogenic strategies.

Immunotherapeutic strategy	Target	Number of animals	Immunologic endpoint	Clinical benefit
Xenogeneic melanoma-antigen-enhanced allogenic tumor cell vaccine	Human glycoprotein 100 (hgp100)	34 dogs	PBMC cytotoxicity Neutralizing anti-hgp100 antibody	1 CR 5 PR 6 SD
Local adenovector human CD40L immunogene transfection	Human CD40L	19 dogs	Circulating cytokines (TNF α , IL8, IL10) Neutralizing anti-human adenovirus serotype 5 antibody	5 CR 8 PR 4 SD

(68–72). While surgical resection is feasible for some dogs with rostrally-confined primary tumors, most canines are diagnosed with invasive inoperable tumors, and hypofractionated ionizing radiation is instituted for local tumor control (73–75). Radiation therapy, alone or as an adjuvant to marginal resection, can achieve satisfactory local primary tumor control (**Figures 1A,B**), however a substantive fraction of dogs will develop metastatic progression within 6–9 months of diagnosis (67, 68, 76). While the most common site for OMM metastases are regional lymph nodes (77, 78), progression of distant metastases within the pulmonary parenchyma can become life-limiting in dogs that have achieved durable local disease control (67) (**Figures 1C,D**), and the institution of adjuvant cytotoxic agents does not definitively yield any survival benefit (74, 79). As such, no standard-of-care adjuvant therapy in dogs with metastatic OMM exists and creates a unique and ethical opportunity to model novel immunotherapeutic strategies that might not be otherwise possible in human patients. Importantly, commercial reagents for the assessment of immunobiologic endpoints including tumoral expression of PD-L1, tumor-infiltrating lymphocytes, and regulatory T cells have recently been validated in canine tissues (**Figures 1E–H**).

Clinical Evidence for Canine OMM Immunogenicity

With conservation of certain tumor-associated antigens in both humans and dogs (80–82), canine OMM has been explored as a relevant tumor model in evaluating various immunotherapeutic strategies, in particular tumor vaccine (30). Both autologous and xenogeneic (tyrosinase) vaccines exert measurable anticancer activities in subsets of dogs treated, with objective responses being documented in patients with unsatisfactorily controlled primary tumors, as well as regression of regional and distant metastatic lesions (83–85). In addition to tyrosinase as a therapeutic target, a limited number of investigations have characterized the immunogenic targeting of xenogeneic GP100 and adenoviral CD40L transfection through vaccination strategies; demonstrating immunobiologic activities and clinical benefit in dogs with OMM (**Table 1**) (86, 87).

In addition to vaccines, checkpoint blockade strategies have been recently described in dogs with OMM. Initial studies

identified the upregulation of PD-L1 following INF- γ exposure in immortalized canine melanoma cell lines, as well as, PD-L1 expression in 100% (8/8) of spontaneous canine OMM samples (88). A follow-up confirmatory study similarly identified 90% (36/40) OMM samples to express PD-L1, and importantly demonstrated that tumor-infiltrating lymphocytes, both CD4⁺ and CD8⁺, expressed PD-1 (89). Expressions of PD-L1 by melanoma cells and PD-1 by TILs, support the potential for melanoma cells to induce T-cell exhaustion as an immunoevasive mechanism. To confirm the functional immunosuppressive activities of PD-L1 expressions in canine OMM, an anti-PD-L1 antibody was evaluated in dogs with OMM, with suggestive evidence for survival time prolongation in four dogs with pulmonary metastasis when compared to historical controls (90). Collectively these clinical investigations support the relevancy of canine OMM as a naturally-occurring model system for testing immunotherapeutic combinations inclusive of other immunomodulatory strategies such as radiation-induced ICD and abscopal activities.

Canine Appendicular Osteosarcoma (OS)

Osteosarcoma (OS) accounts for 85% of all skeletal tumors in the dog with an estimated 10,000 dogs diagnosed each year (33, 91), and is a disease primarily afflicting the appendicular skeleton of large and giant breed dogs (33). Similarly, OS is the most common primary focal skeletal tumor in people, being the third most frequent cause of cancer in adolescents (92). The comparative similarities at genetic, molecular, and clinical levels shared between canine and pediatric OS are robust (12, 13, 33–35, 93–97); evidence that strongly emphasize the potential value for the utilization of canine OS to guide investigations related to pathogenesis and novel therapeutic discovery (98).

The biologic behavior of OS is aggressive, starting within the local bone microenvironment but then involving distant organs because of metastatic progression. Although 15% of dogs and 20% of people present with detectable lung metastases, the development of metastatic foci in the absence of chemotherapy is 90% within 1 year for dogs and 80% within 2 years for people (99, 100). While the institution of chemotherapy for OS patients has tripled the cure rate of people (20 $\rightarrow$ 65%) and doubled the survival time of dogs (130 $\rightarrow$ 270 days), no substantive improvement in long-term outcomes has been achieved for either species over the past 2 decades despite the institution of dose intensification strategies (101, 102). Given the current therapeutic ceiling, there is clinical need to explore alternative adjuvant therapies that might improve metastatic disease control.

Because the cure rate for canine OS remains <10% 3-years post diagnosis (103), the palliative management of primary tumor malignant osteolysis and associated pain is considered an acceptable treatment option in veterinary medicine (104). Similar to skeletal metastasis in humans, ionizing radiation alone or with bisphosphonates is considered effective for attenuating pathologic bone resorption and associated pain syndromes in affected dogs (105–111), and provides a durable therapeutic window of acceptable analgesia lasting from 3

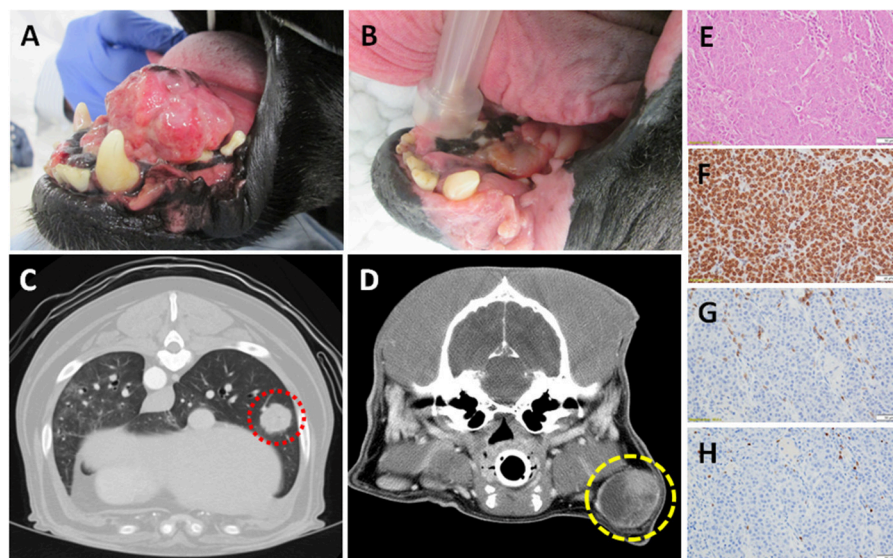


FIGURE 1 | Canine OMM (A) pre- and (B) post- palliative radiation therapy; note the achievement of strong partial response of primary tumor (courtesy of Dr. Michael Kent, UC Davis). Computed tomography of (C) distant pulmonary (red) and (D) regional lymph node metastases (yellow) of OMM origin; demonstrating the potential for reproducible and quantitative volumetric assessments for documentation of abscopal activities. Panel (E–H; top to bottom) represents the histologic and immunobiologic assessment of a regional lymph node effaced with amelanotic melanoma by H&E, PD-L1, CD3⁺ tumor-infiltrating lymphocytes, and regulatory T cells (courtesy of Dr. Jonathan Samuelson, UIUC). Magnification 400x.

to 12 months, whereby it is possible to serially monitor for the development, progression, or regression of distant pulmonary metastases. Prospective assessment of combinatorial strategies inclusive of radiation and other immunostimulatory therapies to amplify tumoral lymphocyte infiltrates such as ICD-inducing anthracyclines, toll-like receptor agonists, and checkpoint blocking antibodies which maximally generate robust abscopal effects could be leveraged to guide translational studies in human patients (Figure 2).

Clinical Evidence for Canine OS Immunogenicity

Scientific and clinical evidence supports OS to be immunogenic in dogs and humans (29, 112), and strategies that amplify anticancer immunity would be expected to improve long-term outcomes. In dogs, investigations have demonstrated immune activation as an effective strategy for either regressing macroscopic metastases or delaying micrometastatic disease progression. For macroscopic disease, inhalation therapy with liposome interleukin-2 demonstrated the capacity to activate immune cells with consequent regression of measurable pulmonary metastases (113, 114). In the setting of microscopic disease, dogs that develop post-operative wound infection after limb-spare surgery experience prolongation to pulmonary metastases development, with survival times being doubled in dogs that develop osteomyelitis (115, 116), and mechanistically localized infectious inflammation has been linked to NK cell and macrophage activation with consequent mediation of systemic anticancer effects (117). Similarly, L-MTP-PE, a synthetic lipophilic glycopeptide capable of activating monocytes and macrophages to a

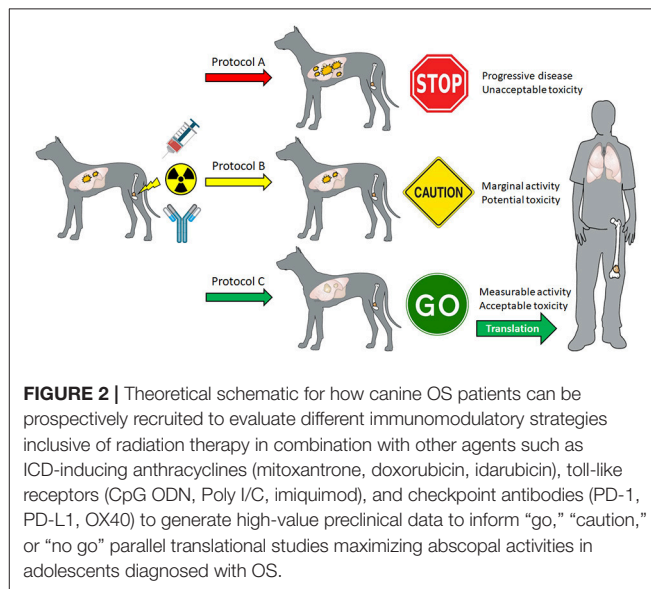


FIGURE 2 | Theoretical schematic for how canine OS patients can be prospectively recruited to evaluate different immunomodulatory strategies inclusive of radiation therapy in combination with other agents such as ICD-inducing anthracyclines (mitoxantrone, doxorubicin, idarubicin), toll-like receptors (CpG ODN, Poly I/C, imiquimod), and checkpoint antibodies (PD-1, PD-L1, OX40) to generate high-value preclinical data to inform “go,” “caution,” or “no go” parallel translational studies maximizing abscopal activities in adolescents diagnosed with OS.

tumoricidal state, when administered to dogs with OS increases survival time, and underscores the key participation of innate immune cell activation for curbing metastatic progression (118, 119). Lastly, intravenous delivery of a genetically modified *Listeria monocytogenes* to OS-bearing dogs exerts promising anticancer immune activities and extends survival times (120). Collectively, these clinical investigations support the feasibility of stimulating immune effector cells to regress macroscopic and

microscopic metastatic disease burdens in dogs diagnosed with OS.

Emerging Abscopal Modeling in Canine OMM and OS

While existing aggregate data for validating radiation-induced ICD and abscopal activities in pet dogs with cancer remains limited, experimental data is emerging to support the prospective evaluation of hypofractionated radiation therapy for augmenting immune responses. Recently, combinatorial strategies inclusive of ionizing radiation, hyperthermia, and intratumorally delivered virus-like nanoparticle-based therapies have been evaluated in canine OMM, and demonstrate the capacity to elicit immunogenic changes within the localized tumor microenvironment including the promotion TILs into the primary tumor (121, 122). In another investigation conducted in dogs with OMM, abscopal effects were documented in dogs treated with a combination of localized radiation therapy, intratumoral CpG ODN, and an indolamine-2,3-dioxygenase inhibitor (123). For canine OS, combining radiation and immunotherapy has been recently explored in a first-in-dog trial of autologous natural killer (NK) cells (124). In this study, OS-bearing dogs were treated with a coarsely fractionated radiation protocol consisting of 9 Gy once weekly for 4 treatments, with NK cells being harvested and expanded, and then delivered back to dogs by intratumoral injection following the completion of radiation therapy. Of the 10 dogs treated, 5 remained metastasis-free at 6 months, and one had regression of a suspicious pulmonary nodule detected at the time of diagnosis.

FUTURE DIRECTIONS AND CONCLUSIONS

Dogs diagnosed with naturally-occurring cancers of comparative relevance can serve as biology-rich models of disease. If

leveraged appropriately, the inclusion of pet dogs can accelerate the discovery of optimal combinations of radiation and immunotherapies which robustly and consistently elicit life-extending abscopal effects. With the availability of linear accelerator-based radiation facilities in veterinary centers analogous to human hospitals, coupled with the development of dog-specific immune-based therapies including vaccines, monoclonal antibodies, and CAR-T technologies, the purposeful inclusion of pet dogs with immunogenic tumors should be seriously contemplated as a unique strategy to aid in defining the limits and benefits of radiation-induced abscopal activities.

The scientific development and clinical assessment of novel immunotherapeutic strategies are rapidly growing areas in veterinary medicine and have demonstrated promise in the settings of canine OMM and OS. Given the conserved biology of these two immunogenic solid tumors between dogs and people, unique opportunities exist collectively for human and veterinary researchers to pilot and validate innovative immune strategies inclusive of radiation therapy in efforts to harness the promise of abscopal anticancer activities.

AUTHOR CONTRIBUTIONS

TF and KS project conception and manuscript authorship.

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Development of Severe Combined Immunodeficient (SCID) Pig Models for Translational Cancer Modeling: Future Insights on How Humanized SCID Pigs Can Improve Preclinical Cancer Research

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Within the last decade there have been several severe combined immunodeficient (SCID) pig models discovered or genetically engineered. The animals have mutations in *ARTEMIS*, *IL2RG*, or *RAG1/2* genes, or combinations thereof, providing SCID pigs with NK cells, but deficient in T and B cells, or deficient in NK, T, and B cells for research studies. Biocontainment facilities and positive pressure isolators are developed to limit pathogen exposure and prolong the life of SCID pigs. Raising SCID pigs in such facilities allows for completion of long-term studies such as xenotransplantation of human cells. Ectopically injected human cancer cell lines develop into tumors in SCID pigs, thus providing a human-sized *in vivo* model for evaluating imaging methods to improve cancer detection and therapeutic research and development. Immunocompromised pigs have the potential to be immunologically humanized by xenotransplantation with human hematopoietic stem cells, peripheral blood leukocytes, or fetal tissue. These cells can be introduced through various routes including injection into fetal liver or the intraperitoneal (IP) space, or into piglets by intravenous, IP, and intraosseous administration. The development and maintenance of transplanted human immune cells would be initially (at least) dependent on immune signaling from swine cells. Compared to mice, swine share higher homology in immune related genes with humans. We hypothesize that the SCID pig may be able to support improved engraftment and differentiation of a wide range of human immune cells as compared to equivalent mouse models. Humanization of SCID pigs would thus provide a valuable model system for researchers to study interactions between human tumor and human immune cells. Additionally, as the SCID pig model is further developed, it may be possible to develop patient-derived xenograft models for individualized therapy and drug testing. We thus theorize that the individualized therapeutic approach would be significantly improved with a humanized SCID pig due to similarities in size, metabolism, and physiology. In all, porcine SCID models have significant potential as an excellent preclinical animal model for therapeutic testing.

Keywords: severe combined immunodeficiency, swine, humanization, cancer, xenograft, pre-clinical, animal model

INTRODUCTION

A new field of personalized medicine has been evolving over the last decade, especially with respect to advances in individualized cancer therapies, ranging from T cell and NK cell immunotherapies, targeted monoclonal antibody therapy, and newly developed small molecule drugs. As progress is made toward the development of cancer therapies, it is critical that preclinical animal models can dependably represent human responses to drugs. Presently, mice are the most commonly used model for preclinical animal drug trials (1). However, many preclinical cancer drug trials that succeed in mice fail in humans due to vast differences in physiology, metabolic processes, and size (2, 3). The drug development process is intensive; on average, 12 years of research and \$1–2 billion is required to bring a new drug to market (4, 5). To maximize the efficiency of preclinical drug and therapy testing, large animal models that better parallel human physiology are needed.

Mice with severe combined immunodeficiency (SCID) are an extremely versatile animal model for the field of cancer biology, although they pose significant limitations. The ability to engraft SCID mice with a human immune and/or cancer cell lines has made them an invaluable model for research (6, 7). Although mice are important for initial studies in different cancer fields, they are often not good models for specific aspects of human oncology (2, 8). Limitations of mouse models of cancer include small size, difficulties in modeling human tumor heterogeneity (9) and metabolic differences to humans (10, 11).

Large animal models can be more costly than murine studies, thus murine studies remain valuable for first line screens. However, testing in larger animal models is warranted to better predict outcomes in human and should be used in follow-up studies as an alternative animal model (12). Immunocompetent and SCID pigs are now being developed for human disease research purposes (13–18). Swine are more similar to humans with respect to size, anatomy, genetics, and immunology, therefore immunodeficient pigs may be a superior animal model for preclinical testing of cancer therapeutics (19–21).

Within the last decade there have been numerous SCID pig models created (16–18, 22–28) or discovered (29, 30). One of the hurdles to working with SCID pigs is maintaining viability due to susceptibility to disease. The use of positive-pressure biocontainment facilities (31) and standard animal isolators (27) have improved SCID pig health and viability. The ability to house immunodeficient pigs in a controlled environment increases their lifespan allowing them to be utilized for long-term biomedical research. Pigs are comparable in size to humans, have

more similar metabolism to humans than mice (32, 33), and can be transplanted with larger human tumors.

In this review we describe the different SCID pig models that have been reported in recent years, as well as published methods established to raise SCID pigs for use in long-term research trials of 6 months or more. We describe the importance of human tumor or cancer cell xenotransplantation and how researchers can utilize immunodeficient pigs for translational studies relevant to human patients. In addition to tumor xenografts, the SCID pig has the potential to be engrafted with a human immune system, or “humanized,” just as numerous SCID mouse models have been humanized. While there is no published research on the development of a humanized SCID pig, substantial progress is being made toward this endeavor. We describe the different methods of humanization that could be used in SCID pigs, including fetal liver and intraperitoneal (IP) injections, as well as intravenous (IV), IP, and intraosseous (IO) injection in piglets. Despite the early developmental stage for humanized SCID pigs, the SCID pig has vast potential to be utilized for translational oncology. Our overarching hypothesis in this review is that porcine SCID models will be more translational than mouse models for oncology research in the future.

EXISTING SCID PIG MODELS

Previously Described and Generated SCID Pigs

Within the last decade, numerous SCID pig models have been developed through mutagenesis or discovery of natural mutations. These SCID pig models are outlined in **Table 1**. **Figure 1** shows the genetic and molecular mechanisms for the mutations described below that cause SCID, and **Figure 2** shows the differentiation step blocked by each of these mutations.

The first SCID pig was described in 2012 (13) after a serendipitous discovery in an infection study (29). To confirm the lack of a functional immune system, these SCID pigs were transplanted with human cancer cell lines. Injected cells were not rejected and developed into tumors in the SCID pigs (13). After further analysis, it was found that the discovered SCID pigs had two naturally occurring mutations in two separate alleles within the *Artemis* (*DCLRE1C*) gene, which leads to SCID either in the homozygous or compound heterozygous state (30).

Artemis is required for DNA repair during T and B cell development. Specifically, during the process of VDJ recombination, after RAG1/2 nucleases cleave DNA at the RSS sequences flanking V, J (and sometimes D) segments (34), a hairpin loop then forms at the end of the double stranded break (DSB). Ku70/80 proteins are recruited to the area of the DSB along with Artemis protein, which is responsible for cleaving the hairpin loop so it can be ligated by Ligase IV (35). Without functional Artemis, these hairpins are not cleaved, and functional V, D, and J joins cannot be made. Lack of Artemis function leads to a cellular profile in which T and B cells are deficient, but NK cells develop ($T^- B^- NK^+$) and are functional (29, 30, 36). Homozygous or compound heterozygous *Artemis* pigs can be

Abbreviations: SCID, severe combined immunodeficiency; IL2R γ , Interleukin 2 receptor gamma; RAG, recombination activating gene; Hematopoietic stem cells, HSCs; PBL, peripheral blood leukocytes; VDJ, variable, diversity, and joining; TCR, T cell receptor; BCR, B cell receptor; DSB, double stranded break; NSG, NOD-SCID-IL2R γ ; IP, intraperitoneal; IV, intravenous; TREC, T cell receptor excision circles; GVHD, graft vs. host disease; BLT, bone marrow, liver, and thymus; CAR, chimeric antigen receptor; CRS, cytokine release syndrome; PET, positron emission tomography; MRI, magnetic resonance imaging; CT, computer tomography; US, ultrasound; PDX, patient derived xenograft; CDX, cell derived xenograft.

TABLE 1 | Previously described SCID pig models.

Mutation(s)	Mutagenesis method	Cellular phenotype	Breed	Rearing method	Oldest age reported	References
<i>IL2RG</i>	Gene targeting vector	T [−] B ⁺ NK [−]	Landrace × Large White	Conventional housing	54 days	(16)
<i>ARTEMIS</i>	Natural	T [−] B [−] NK ⁺	Yorkshire	Biocontainment bubble	6 months	(29, 30)
<i>IL2RG</i>	Zinc finger nuclease	T [−] B ⁺ NK [−]	Cross bred	Did not rear	neonatal	(18)
<i>RAG 1/2</i>	TALENs	T [−] B [−]	Bama miniature	Conventional housing	29 days	(22)
<i>RAG 1</i>	Gene targeting vector	T [−] B [−]	Duroc	Did not rear	neonatal	(23)
<i>RAG 2</i>	TALENs	T [−] B [−] NK ⁺	Minnesota minipig	Conventional housing	29 days	(24)
<i>IL2RG</i>	CRISPR/Cas9	T [−] B ⁺ NK [−]	Cross bred	Conventional housing	12 days	(17)
<i>RAG 2</i>	Gene targeting vector	T [−] B [−] NK ⁺	Cross bred	Conventional Housing	12 weeks	(25)
<i>RAG 2 IL2RG</i>	CRISPR/Cas9	T [−] B [−] NK [−]	Yorkshire cross breed	Gn Isolator	34 days	(26)
<i>IL2RG</i>	Zinc finger nuclease	T [−] B ⁺ NK [−]	Cross bred	Isolator	103 days	(18, 27)
<i>ARTEMIS IL2RG</i>	Natural and CRISPR/Cas9	T [−] B [−] NK [−]	Yorkshire	Biocontainment bubble	18 days	(28)

raised to 6 months of age in biocontainment facilities developed at Iowa State University [31, unpublished observation].

Another SCID pig was also described in 2012 with an engineered mutation within the *IL2RG* gene (16). In humans and mice, the IL2 receptor γ (IL2R γ) subunit is required for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 signaling (37). The *IL2RG* gene is on the X chromosome in mammals and the receptor is expressed on lymphoid cells, including developing cells. The cytokines noted are required for proper lymphoid development, and thus deletion of the IL2R γ subunit disrupts development of T and NK cells, and B cells to a variable extent (38, 39). The cellular phenotype of these *IL2RG* knockout pigs was T[−] B⁺ NK[−], similar to humans (38, 39). B cells in *IL2RG* knockout SCID pigs were not able to secrete immunoglobulin nor class switch due to absence of helper T cells (16). Interestingly, cloned heterozygous *IL2RG*^{+/−} females exhibited SCID-like phenotypes, which was attributed to aberrant X-inactivation. These females were raised to sexual maturity and crossed with WT males; female *IL2RG*^{+/−} offspring from this cross phenotypically resembled WT animals (16). This finding emphasizes the importance of monitoring for SCID phenotype status in cloned piglets even if the expected outcome is a carrier animal. Other groups have also introduced mutations in the *IL2RG* gene by CRISPR/Cas9 (17) and zinc finger nuclease (18) methods, and the resulting pigs also displayed cellular phenotypes of T[−] B⁺ NK[−]. Animals in these studies were raised in conventional settings and had lifespans that ranged from 12 days to 7 weeks (16–18).

The recombination activating genes, *RAG1* and *RAG2*, have previously been mutated to create pig models. They code for subunits of a nuclease (RAG1/2), that is involved in VDJ recombination required for T and B cell receptor (TCR and BCR, respectively) generation (40). Without functional RAG1/2 nuclease, VDJ recombination does not initiate, and T and B cells do not develop (41, 42). Homozygous or biallelic *RAG1* or *RAG2* SCID pigs lacked IgM⁺ B cells and CD3⁺ cells in peripheral blood (22, 23, 25). NK cells were present in these animals and were classified as either CD3[−] CD8 α ⁺ (22) or CD16⁺ CD8 α ⁺ (25). RAG knockout pigs were generated with either TALENs (22, 24), gene targeting vectors (25), or CRISPR/Cas9 (26)

mutagenesis methods. Previous *RAG1* or *RAG2* mutant SCID pigs have been raised to 29 days (22, 24) to 12 weeks (25) of age in conventional housing.

Once single mutant pigs were established, research groups began to introduce mutations in both VDJ recombination pathway genes (*RAG1/2* or *ARTEMIS*) and *IL2RG* to produce pigs that lacked innate and adaptive immune function, generating T[−] B[−] NK^{−/lo} SCID pigs (26, 28). Double-mutant pigs are an important animal model to develop, as rodent models of SCID mice lacking NK cells, as well as T and B cells, engraft human cells better than T[−] B[−] NK⁺ models (43). It is therefore of interest to generate a T[−] B[−] NK[−] SCID pig model for humanization studies. In 2016, *RAG2/IL2RG* knock out piglets were generated and used in pathogenesis study with human norovirus (26). *RAG2/IL2RG* SCID pigs lacked T and B cells, and there were decreased numbers of NK cells compared to controls. The presence of some NK cells was attributed to a hypomorphic mutation within IL2R γ (26). Our group has recently engineered a complete *IL2RG* knockout that was introduced into an *ARTEMIS* null genetic background resulting in SCID pigs that lack T, B, and NK cells (28).

Methods for SCID Pig Rearing

One of the difficulties to overcome when using SCID pigs in research is maintaining animal viability. SCID pigs raised in conventional settings typically succumb to disease between 6 and 12 weeks of age [unpublished observation, 17]. Biocontainment facilities have been specifically designed to limit exposure of Iowa State University's *ARTEMIS*^{−/−} SCID pigs to any micro-organisms (Figure 3A). These rooms have positive-pressure HEPA filtered air flow into a containment bubble and all water entering the bubble is UV irradiated and filtered through a 0.5 μ m filter. Personnel entering the bubble wear appropriate garments to limit introduction of organisms into the room, including room dedicated protective suits, hair net, surgical mask, gloves and rubber boots (31). Piglets are derived either by snatch farrowing (caught in a sterile towel as they are delivered vaginally) or by cesarean section and are transferred immediately into

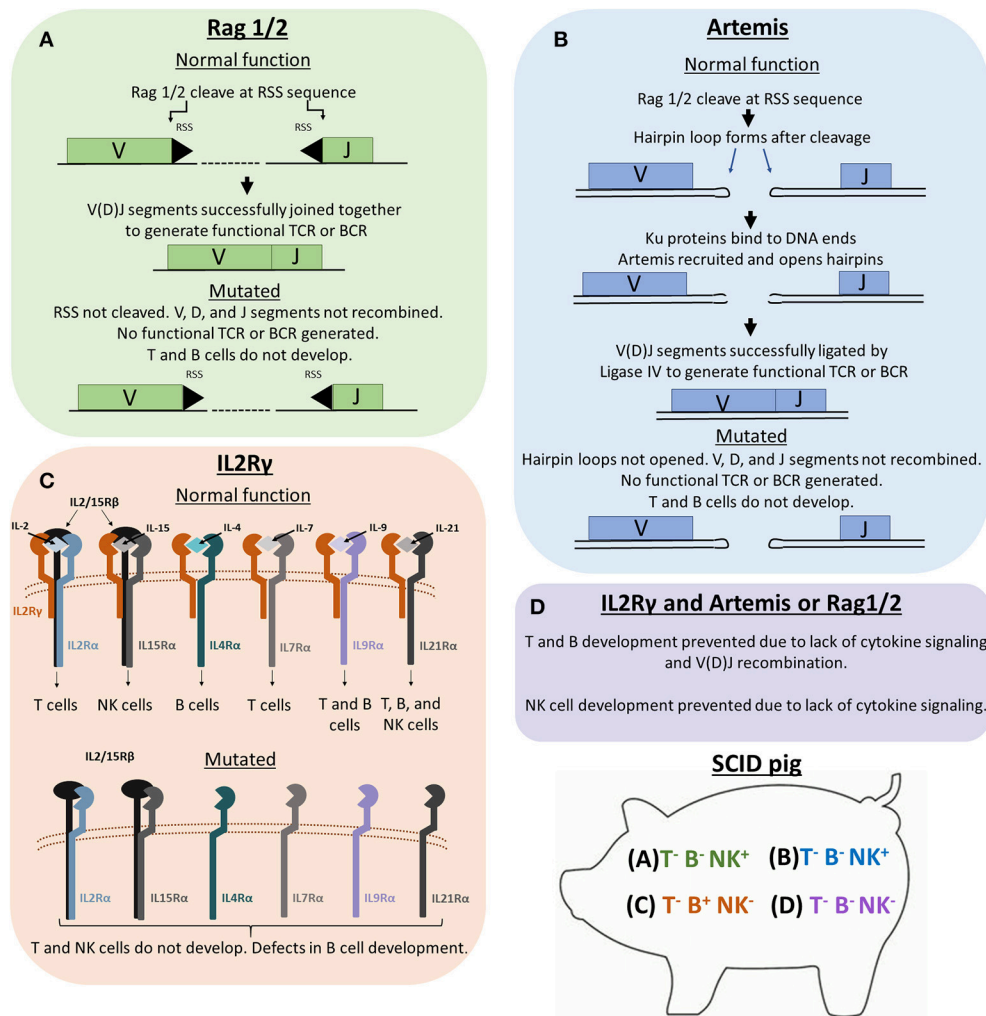


FIGURE 1 | Genetic and molecular mechanisms of Rag1/2, Artemis, and IL2R γ in lymphoid development. Previous SCID pig models have been generated or described with mutations in *ARTEMIS*, *RAG1*, *RAG2*, or *IL2RG*. **(A)** Rag1 and 2 are subunits of an endonuclease that cleave recombination signal sequences (RSS) flanking V, D, and J gene segments. Cleavage of RSS sequences are required for the gene segments to be joined together. Non-functional Rag1 or 2 proteins cannot cleave these sequences, therefore preventing T cell receptors (TCRs) and B cell receptors (BCRs) from forming. T cells and B cells cannot develop due to non-functional TCR and BCR rearrangement. **(B)** Artemis is an endonuclease that is responsible for the cleavage of hairpin loops that form after Rag1 and 2 cleaves RSS sequences. These hairpin loops must be cleaved in order for Ligase IV to ligate V, D, and J gene segments together. If Artemis is not functional, these hairpin loops cannot be cleaved, which prevents TCR and BCR rearrangement. **(C)** IL2R γ is a subunit required in the receptors for IL-2, IL-15, IL-4, IL-7, IL-9, and IL-21. Without functional IL2R γ , developing cells that require these cytokines for development (mainly T, B, and NK cells) are not receptive to cytokine signaling, which prevents proper differentiation of T, B, and NK cells. **(D)** Pigs with mutations in both a VDJ recombination gene (*RAG1/2* or *ARTEMIS*) and *IL2RG* lack T, B, and NK cells.

a sterilized bubble. Piglets are immediately fed pasteurized colostrum for the transfer of maternal immunoglobulin (44), fed sterile milk replacer for 21 days, and then transitioned to irradiated feed, which is continued throughout life (31). Specific pathogen-free (SPF) *ARTEMIS*^{+/−} carrier females have been raised to sexual maturity and are able to naturally farrow *ARTEMIS*^{−/−} SCID litters within the ISU bubble facilities (Figure 3B). *ARTEMIS* mutant SCID pigs can be successfully reared to 6 months of age in these facilities (unpublished observation).

Survivability of previous *IL2RG* knock out pigs has varied from 2 to 7 weeks (16) and derivation of animals and available housing likely impacts outcome. Recently Hara et al. (27) used small isolators and developed piglet delivery protocols to help extend the lifespan of *IL2RG* knock out SCID pigs. To achieve this goal, excised uteruses were brought into isolators units, piglets were delivered, and reared within these isolators. One SCID piglet raised in the isolators was raised to a planned endpoint of 12 weeks of age without incidence of bacterial or fungal disease (27).

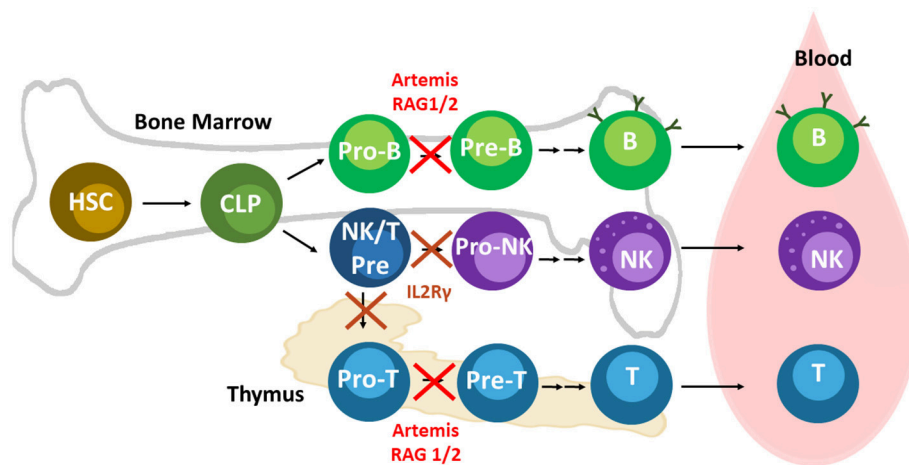


FIGURE 2 | Lymphoid development and relevant SCID pig mutations. Mutations in Artemis, RAG1/2, and IL2R γ leads to SCID in pigs. Artemis and Rag1/2 are active in Pro-B and -T cells during differentiation. IL2R γ is required at an earlier stage of development than RAG1/2 and Artemis. NK cells and T cells both require cytokine signaling through IL2R γ early in differentiation. Mutations in IL2R γ prevent differentiation of T and B cells. Mouse B cells appear to rely on IL2R γ signaling more than human and pig B cells. B cells can still develop in humans and pigs with mutations in IL2R γ , although they are mostly non-functional due to the absence of helper T cells.



FIGURE 3 | Biocontainment facilities for rearing SCID pigs. **(A)** Biocontainment facilities for the rearing of *ARTEMIS*^{-/-} SCID pigs. **(B)** SPF female *ARTEMIS*^{+/-} carriers nursing 3 week old SCID and non-SCID piglets after naturally farrowing in biocontainment facilities.

SCID PIG CANCER XENOTRANSPLANTATION STUDIES

Existing Immortal Cell Lines Develop Into Tumors in SCID Pigs

Since the generation of SCID pigs is so recent, there are only a few studies that have been published on the ability of SCID pigs to accept human xenografts. The first SCID pig xenograft study involved the transplantation of human melanoma (A375SM)

and pancreatic carcinoma cell (PANC-1) into the ear tissue of *ARTEMIS*^{-/-} SCID pigs (13). All SCID pigs receiving cancer cells developed tumors at the site of injection, thus establishing an orthotopic model of melanoma that could be studied further (13). Additionally, the ability of ovarian carcinoma cell line OSPC-ARK1 to develop tumors in *ARTEMIS*^{-/-} SCID pigs was explored. SCID pigs were injected in the ear and neck muscles with OSPC-ARK1 cells and subsequently monitored for tumor development. In 3 of the 4 SCID pigs injected, tumors developed

within 30 days, with a shortest time of 7 days to palpable tumors. Biopsy samples revealed the ovarian tumors in SCID pigs expressed diagnostic markers commonly used in human cancer diagnoses, and tumors in SCID pigs resembled human tumors (45).

Pigs biallelic for *RAG2* mutations can engraft human induced pluripotent stem cells (iPSCs). Injected iPSCs developed teratomas that represented endoderm, mesoderm and ectoderm tissues (24). Teratomas were grossly visible 12 days after cell inoculation for one recipient; and about 7.5 weeks in the other recipient. Histological analysis revealed CD34⁺ and CD45⁺ cells developed in the teratoma, (24), indicating that human immune lineage can survive and differentiate in *RAG2* knockout pigs. This important finding indicates that SCID pigs can accept various types of human xenografts. In a follow-up study, *PERFORIN*, and *RAG2* double knock out (*Pfp/RAG2* dKO) mice and *RAG2* knock out pigs were compared for their ability to engraft human iPSCs. The *RAG2*^{-/-} pigs developed teratomas from injected iPSCs at a higher rate than the *Pfp/RAG2* dKO mice. Human teratomas that developed in the *RAG2* knockout SCID pigs also had a higher prevalence of CD45⁺ and CD34⁺ cells in the teratoma than in SCID mice (46). Thus, the *in vivo* environment in pigs supports the growth and differentiation of human cells, and in some instances, is an improved system over SCID mice.

PORCINE IMMUNOLOGICAL SIMILARITIES TO HUMAN

Several aspects of the pig immune system are more similar to humans than mice, providing another advantage of swine models for research (39). Humans and pigs have higher sequence orthology for immune-related genes (termed the “immunome”) than humans and mice (20). Immunome-specific gene family expansions, a measure of evolutionary divergence, have occurred in pig relative to human at half the rate detected in mouse or cow (20), and pigs have significantly fewer unique genes not found in humans when compared to unique gene abundance in cow or mouse (Figure 4). Additional analyses have further expanded human and pig similarities, although absence of two inflammasome gene families have also been found uniquely in the pig genome (47). As well as immunome structural similarities, immune responses are highly comparable between human and pig [reviewed in (41)]. For example, the transcriptomic response to lipopolysaccharide of pig macrophages *in vitro* is more similar to human responses as compared to mice. Specifically, clusters of genes with IDO1 as hub were detected in human and pig macrophage responses, but not in mice, while a NOS2A-related gene cluster was only found in the mouse macrophage LPS response (48).

Human hematopoietic stem cell (HSC) development in swine for humanizing pigs will be dependent on swine cytokine signaling. Hence, it is important to determine the cross reactivity of porcine cytokines with human cells. Protein sequence analysis shows that swine share more homology in cytokines involved in hematopoiesis with humans than mice (Figure 5; Supplemental Table 1), which suggests that certain human

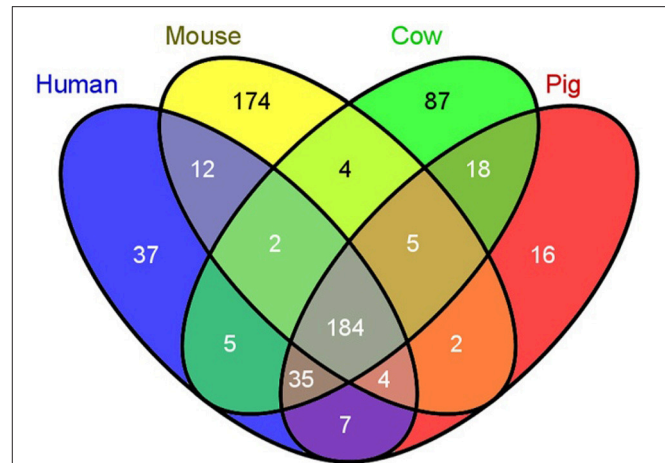


FIGURE 4 | Swine have fewer unique immunological genes compared to humans than do mice. A comparison of the number of unique immunological genes was compared between humans, mice, cows, and pigs. Pigs have 2 times less unique genes, while mice have 4.7 times more unique genes compared to humans [Reprinted from Dawson et al. (20); Figure 1].

lineages may differentiate with greater success in SCID pigs than in SCID mouse models.

ROUTES FOR HUMANIZATION AND APPLICATIONS

Given the high similarity of swine and human immune genes, we would anticipate that human HSCs transferred into SCID pigs would successfully engraft and differentiate into representative human immune cell types. Current building of swine SCID models relies heavily on translating methods used for mouse humanization to generate new humanized SCID pig models. To humanize the mouse, three different approaches are utilized (6, 7). These methods include transfer of purified human CD34⁺ stem cells, peripheral blood leukocytes (PBLs), or transfer of fetal bone marrow, liver, spleen, and lymph node tissues. Just as in SCID mouse models, these same approaches and cell types can be investigated as methods to humanize SCID pigs. The pig immune signaling molecules that support engraftment are expected to be similar to humans, thus we expect successful development of human immune cells.

Currently the NOD-SCID-IL2R γ (NSG) knockout mouse is the gold standard model for humanization. The *Sirpa* allele in the NOD background contains polymorphisms that allow the encoded Sirpa protein to bind to human CD47, which then sends a inhibitory signal that prevents phagocytosis of human cells (49, 50). Swine SIRPA also binds to human CD47 (51), so we speculate that porcine SIRPA-dependent phagocytosis of human cells would not be a barrier to SCID pig humanization.

The following sections describe previous humanization methods performed in SCID mice and other large animal models, and how these methods can be utilized to humanize SCID pigs. Figure 6 shows an overview of different human immune cell

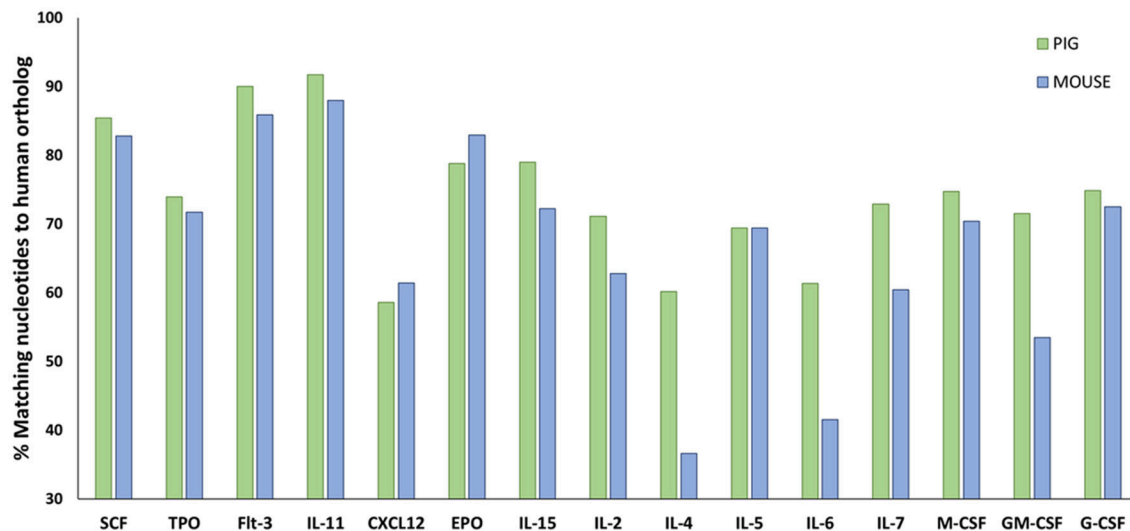


FIGURE 5 | Amino acid sequence comparisons in hematopoietic cytokines for pig and mouse compared to humans. Amino acid sequences for relevant hematopoietic cytokines and other ligands were acquired from Ensembl (<https://useast.ensembl.org/index.html>). The percentage of matching sequence between humans to pigs and mouse is shown above. Porcine shares higher sequence similarity to humans for a majority of hematopoietic cytokines compared to mice. **Supplemental Table 1** shows the accession numbers from the sequences that were compared.

types and anatomical injection sites for SCID pig humanization. Past studies utilizing injection of human HSCs or human induced pluripotent stem cells into large animal models are presented in **Table 2**.

CD34⁺ Cell Injection via Fetal Liver and Intraperitoneal Space

Successful humanization of SCID pigs will require that human HSC be injected into sites of hematopoiesis in the pig. During gestation the initial location of hematopoiesis is the yolk sac (58). As gestation continues, the fetal liver becomes the site of hematopoiesis, typically around the beginning of the second trimester (59–63). During swine gestation, hematopoiesis begins at day 30 in the fetal liver (62). Intrauterine injection of human hematopoietic cells during the fetal liver phase of hematopoiesis would provide a rich environment for human stem cells to engraft and differentiate (64), as supporting cells in the fetal liver niche express c-Kit, CD34, CXCL12, and NOTCH (59). Additionally cell subsets in the fetal liver can promote hematopoiesis, such as CD34^{lo} CD133^{lo} cells that have been described in human (65). Differentiated human cells that develop in the SCID pig liver may also migrate to the bone marrow around the same time as other developing swine immune cells, which may increase the ability of human immune progenitors to engraft within the SCID pig bone marrow. Fewer human cells would be required for the fetal liver injection strategy when compared to the number of cells required to engraft a fully developed piglet. Taken together, we hypothesize that fetal injection of human hematopoietic stem cells will likely lead to the highest levels of engraftment compared to other methods described in later sections.

The first study involving *in utero* injection of human cells into a large animal was performed by Zanjani et al. (52). Human fetal

liver cells were injected into the IP space of fetal sheep at days 48–54 gestation (145 day term) through the uterine wall. The recipient sheep were immunocompetent, but pre-immune at this stage of development. Two of the derived sheep were raised to 15 months of age, and human CD3⁺, CD16⁺, and CD20⁺ cells were still in circulation, albeit at very low frequencies (52). Other studies involving the transplantation of human CD34⁺ cells in the fetal liver of pre-immune sheep have resulted in similarly low levels of human cell engraftment and differentiation (55, 57).

In addition to sheep, *in utero* injection of human CD34⁺ cells have been performed in pre-immunocompetent conventional pigs. The first was described in 2003 (53) with the injection of cord blood derived CD34⁺ cells into the IP space of pre-immune fetal piglets at ~40 days of gestation (114 day term). Populations of human CD3⁺ cells were detected in the thymus, CD19⁺ cells and myeloid cells also developed *de novo* in the pig, in as short as 40 days post-injection. Additionally, human CD34⁺ CD45⁺ cells were isolated from pig bone marrow 120 days after transplantation and were subsequently transplanted into SCID mice with successful engraftment of human cells observed. This result indicates that the pig bone marrow environment is able to support the development of functional human HSCs (53).

Humanization of pigs could serve as a source of human T cells for immunotherapeutic use. Ogle et al. (56) depleted CD3⁺ cells from human bone marrow or cord blood and injected into the IP space of fetal piglets at 40–43 days of gestation. Human T, B, macrophages, and NK cells were detected in peripheral blood of piglets using RT-PCR by amplification of CD3, CD19, CD14, and CD16/CD56, respectively. In order to determine if the human T cells had developed *de novo*, blood was analyzed for the presence of human T cell receptor excision circles (TREC). Human TRECs were observed at a level that suggested new human T cells had developed in the swine thymus (56). Similar,

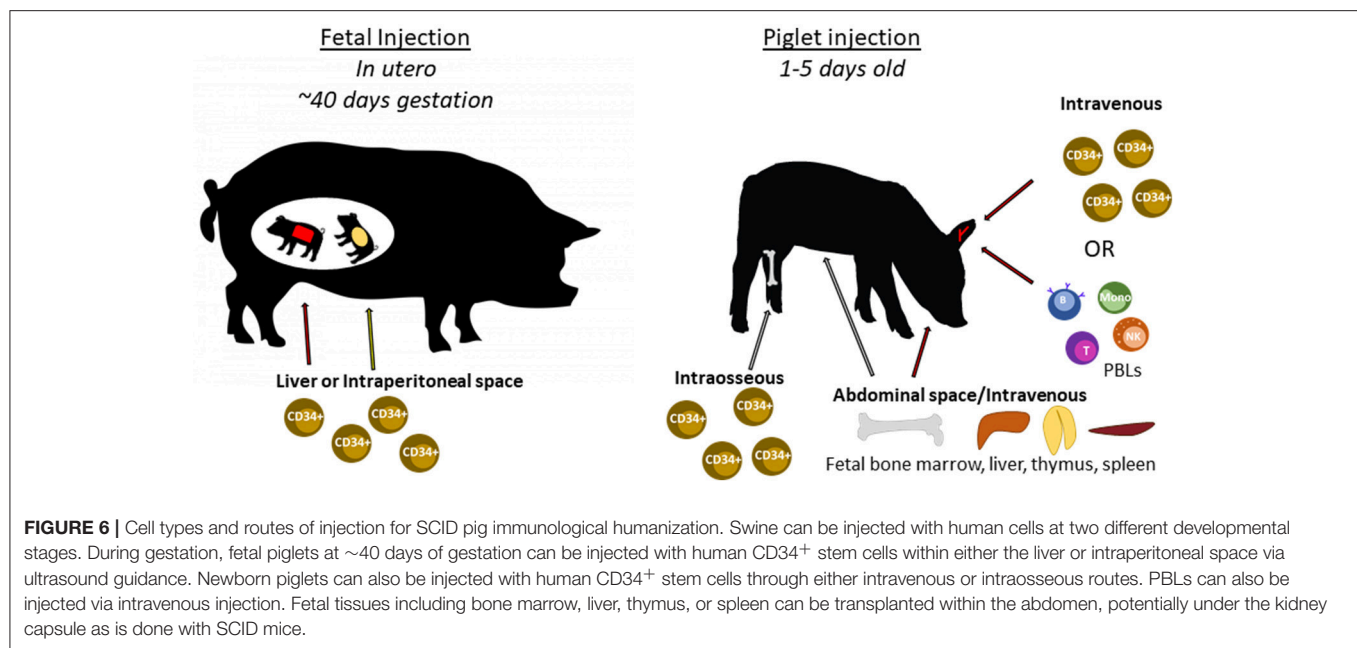


TABLE 2 | Previously described human stem cell injection studies in swine and sheep.

Large animal	Type of human cells injected	Number of cells injected	Age and injection site	Human cell type(s) that differentiated	References
PI sheep	Fetal liver cells	$0.2-1 \times 10^{10}/\text{kg}$	Fetal peritoneal cavity	T, B, NK, myeloid, erythroid	(52)
PI pig	CD34 ⁺ cells from cord	$0.5-3 \times 10^6$	Fetal intraperitoneal cephalad space	T, B, NK, Myeloid	(53)
PI pig	T cell depleted bone marrow	5×10^7 ($5 \times 10^9/\text{kg}$)	Fetal peritoneal cavity	B	(54)
PI sheep	CD34 ⁺ cells from bone marrow	3.6×10^6	Fetal peritoneal cavity	T, B, myeloid, dendritic cells, erythroid	(55)
PI pig	T cell depleted bone marrow or cord blood	5×10^7 ($5 \times 10^9/\text{kg}$)	Fetal peritoneal cavity	T, B, NK, myeloid	(56)
PI sheep	ESC-derived CD34 ⁺	3×10^6	Fetal peritoneal cavity	T, B, NK, Monocytes, neutrophils	(57)
SCID pig	iPSCs	$5-10 \times 10^6$	Piglet ear and lateral flank	CD34 ⁺ , CD45 ⁺	(24)
SCID pig	iPSCs	10×10^6	Piglet ear and lateral flank	CD34 ⁺ , CD45 ⁺	(46)

PI, Pre-immunocompetent; ESC, embryonic stem cells; iPSC, induced pluripotent stem cells.

studies were performed in which fetal swine were injected with human T cell depleted bone marrow (54) or T cell depleted cord blood (66), in which human cell engraftment was observed. In all, these studies show that human T cells can develop *de novo* when human HSC are injected into fetal swine.

Successful engraftment of SCID pigs utilizing *in utero* injections requires consideration of timing and surgical procedures. We hypothesize that a humanized SCID pig could be developed via *in utero* injection of human CD34⁺ cells within the fetal liver or IP space at ~40 days of gestation. We have described detailed laparotomy protocols that can be followed for procedures involving stem cell injection into fetal IP space and livers [(67); Figure 7]. The level of human cell hematopoiesis in a SCID pig model has yet to be determined, however it is expected that engraftment would be comparable to that described for immunocompetent animals. Given the lack of pig immune cell development in pigs with SCID, the available niches for human progenitor cells to develop in the bone marrow and thymus would be increased.

Peripheral Blood Leukocyte Injection via Intravenous or Intraperitoneal Routes

In 1988, the first humanized mouse models were generated in efforts to investigate the AIDS virus interaction with its human host. One of these models described the injection of human PBLs into the IP space of SCID mice (68). Mice were injected by the IP or IV routes with 10–90 million human PBLs (termed hu-PBL SCID mice). IV injection was deemed ineffective in mice, likely due to the difficulty of proper IV administration in a mouse. Human cells injected IP in mice were able to migrate to the spleen, lymph nodes, and were also detected in peripheral blood; 4 weeks post IP injection very few human PBL were detected in the peritoneal space. Mice were vaccinated with tetanus toxoid, which PBL donors were known to be immune. Eight of 10 animals injected with PBLs produced human immunoglobulin against tetanus toxoid which supported that human helper T cells and B cells were functional in the hu-PBL SCID mice. Human CD14⁺ monocytes were also present in the spleens of mice 8 weeks post transplantation (68). These hu-PBL SCID

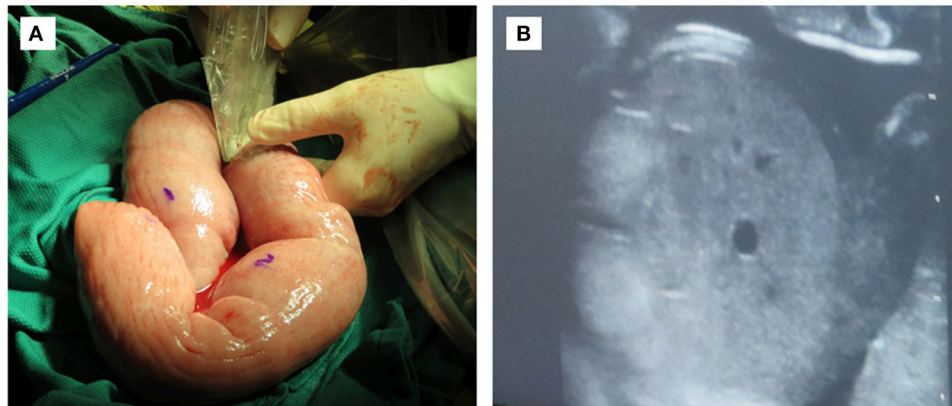


FIGURE 7 | *In utero* injection of fetal intraperitoneal space via laparotomy. **(A)** Exteriorized uterus at 40 days gestation being ultra-sounded for fetuses. Water soluble marker can be used for marking fetuses. **(B)** Ultrasound image of fetal liver with which human CD34⁺ stem cells would be injected.

mice are utilized in a variety of different fields including HIV (69–71), cancer (72, 73), basic immunology (74, 75), and atopic dermatitis (76).

Hu-PBL-SCID pigs could be generated by IV or IP injection of human PBLs into SCID pigs. IV injection of human cells have been deemed ineffective for engraftment in mice. However, tail veins are typically used in mice, which are difficult to properly inject. Piglets have large and visible ear, cephalic, and saphenous veins that are easily accessible. A limitation of human PBL injections in pigs could be the amount of cells required relative to the number of cells injected into mice. Mice are typically 20 g (0.02 kg), while a typical newborn piglet weighs about 1–2 kg. In previous studies, the minimum amount of human PBLs injected into mice is about 10 million cells, which scales up to 0.5–1 billion in a piglet. However, there are strategies to overcome the cell number limitation. One source for human PBLs could be leukoreduction system chambers (LRSCs), which are utilized by blood banks to remove PBLs during plateletpheresis. During a normal collection of platelets from a donor, ~2 billion PBLs can be obtained from LRSCs (77). Another approach is matching a human with a SCID pig and performing repeat PBL injections from the same human donor. Also, it is possible that the number of human PBLs required for successful engraftment of SCID pigs would not be as high as calculated from murine studies. Given that methods to obtain large numbers of PBLs are available, the number limitation is not expected to prevent development of a hu-PBL-SCID pig.

One consideration for using a SCID pig injected with human PBLs is that these animals will eventually develop graft vs. host disease (GVHD). SCID mice injected with human PBLs develop GVHD ~3–11 weeks after injection (78) while it takes 14–16 weeks in SCID rats (79). It is currently unknown how long it would take SCID pigs to develop GVHD after human PBL cell transplantation, as well as how the cellular dose would impact the GVHD time frame. This is a question that will need to be addressed as this model is developed. Another important question that will need to be addressed in developing this model

is the time period required for human PBL engraftment within the SCID pig.

One benefit of the PBL model is that it could be used for short term studies in SCID pigs. SCID pigs raised in conventional settings can typically survive to 6 weeks of age. If piglets are injected with human PBLs shortly after birth (1–5 days), this would give researchers ~a 6 week window to perform experiments. It may also be appropriate to administer immunosuppressive drugs during this period of time to reduce the effects of GVHD.

CD34⁺ Cell Injection via Intraosseous or Intravenous Routes

Another route for humanization is through the injection of purified human CD34⁺ HSCs into live-born piglets. We have previously performed bone marrow transplantations (BMT) on our SCID pigs through IV injection of unfractionated pig bone marrow cells (80). One hypothesis is that human HSC could be administered in the same way to generate a humanized SCID pig. Typically in pig to pig bone marrow transplants, it takes ~10 weeks to observe a moderate increase in the number of circulating porcine lymphocytes (80). We hypothesize that human engraftment and *de novo* development of human cells would require at least 10 weeks to observe human cells in circulation based on pig to pig BMT observations. It may be of value to compare cell dosages and engraftment rates of human and pig HSC in SCID pigs. IV injection of human HSC is much less invasive than fetal injections, however it may take longer to achieve engraftment and differentiation of human cells.

Another method of human HSC administration is through intraosseous (IO) injection. IO injection of stem cells and mesenchymal stem cells have previously been performed in SCID mice (81), dogs (82, 83), and pigs (84). IO injection is also a method for bone marrow transplantation in humans (85). It is hypothesized that IO injections are preferable over IV injections due to stem cell trapping in pulmonary tissue, which is often observed in IV injections (86, 87). In addition,

IO administration introduces cells to the site within which they would differentiate. Protocols have also been developed for the delivery of various substances through IO injection in swine (84, 88, 89). IO injection of human CD34⁺ cells into SCID pigs is therefore another potential route for studying engraftment and humanization models.

Implantation of Human Fetal Bone Marrow, Thymus, and Liver Tissues

Another potential method for humanization of SCID pigs is through the transplantation of human fetal liver, thymus, lymph node, and spleen tissue, as has been previously performed in mice (90). Such human lymphoid tissues can be transplanted into mice either by implantation under the kidney capsule or IV injection of a cellular suspension. Mice transplanted with human lymphoid tissues appear to have immunological protection, as the lifespan of transplanted mice can be extended to 17 months, compared to 4 months for non-transplanted mice. Mice injected with both human thymic and fetal liver cells developed human T and IgG secreting B cells (90). The chimeric mice with human bone marrow, liver and thymus (BLT) are used to study interactions between human immune cells and patient derived melanomas (91).

De novo development of human T cells within the pig requires that human T cells can differentiate within the swine thymus. Transplantation studies show that the porcine thymus supports human T cell development, as mature human T cells develop in athymic mice transplanted with porcine thymus and human HSCs (92, 93). Human T cell development within the swine thymus is particularly important for long term studies because this would allow newly differentiated human T cells to develop tolerance to pig antigens. Human thymic tissue could also be transplanted into SCID pigs for human HLA restricted T cell development. Development of GVHD is observed in mice humanized with fetal bone marrow, liver, and thymic tissue (94), potentially due to human thymus dependent T cell development. Depending on the experimental question being addressed, transplantation of a human thymus may be a preferred method in humanizing SCID pigs.

One issue with generating BLT humanization models is the limited fetal tissue availability, as well as ethical implications. Smith et al. described a way to circumvent these issues by propagating and expanding BLT tissues in one mouse and then transplanting into 4–5 other mice (95). SCID pigs could be useful in this regard as human tissues would have the potential to grow to a large enough size that they could be transplanted again into a second set of animals.

FUTURE OUTLOOK ON THE UTILIZATION OF SCID PIGS FOR CANCER THERAPIES AND RESEARCH

Humanized SCID Pigs for CAR-T and CAR-NK Cell Therapy Research

Chimeric antigen receptor (CAR) T and NK cells have been developed in recent years as a cancer immunotherapy. CAR-T

cells targeted against CD19 for patients with B cell lymphomas and leukemias (96) have been approved by the FDA for therapeutic use (97, 98). One of the issues associated with CAR-T cells is that they can persist and be activated for long periods of time in the body, causing cytokine release syndrome (CRS). Symptoms of CRS manifest as fatigue, fever, nausea, cardiac failure, among other symptoms (99). CAR-NK therapies are being developed to overcome some of the issues associated with CAR-T cell therapy. Protocols have been developed to isolate NK cells from cord blood and expanded for use in patients. NK cells do not persist for long periods of time *in vivo* after infusion (100), do not cause GVHD, and can recognize tumor targets through intrinsic receptors (101). If SCID pigs can successfully develop human NK cells *de novo*, humanized SCID pig blood could be a source of NK cells. Six month old ARTEMIS^{-/-} Yorkshire SCID pigs are ~85 kg (personal observation), and thus according to IACUC guidelines, up to 1.2 L of blood could be collected for human NK cell isolation and used for CAR therapy research.

We envision several applications for a hu-PBL SCID pig in testing cell-based immunotherapies. As more CAR therapy targets are generated, it may be possible to test their efficacy and safety in a humanized SCID pigs that are xenografted with a human tumors. Other CAR therapies that are currently under development are CAR-T cells targeting CD20 (102), CD30 (103), CD33 (104), CD7 (105), and CAR-NK cells targeting CD33 (106) and CD19 (107). In addition, as the field of precision medicine continues to grow, a patient's tumor could be xenografted into a SCID pig and a therapy could be tested. Tumors in SCID pigs could be grown to a comparable size to those found in humans and would therefore be a more representative model compared to the limited size of tumors in mouse models. Similar, studies have been performed in hu-PBL-mice, in which interactions between human thyroid tumors and PBLs were studied (108).

Improving Targeting Imaging Techniques

Pigs are an excellent animal model for surgical and clinical imaging research. Due to their larger size, techniques that are used for humans in the clinics (PET, MRI, CT, US) can also be readily adapted for use in swine. There are immunocompetent pig models of cancer that exist with inducible mutations in p53 (15, 109, 110) and KRAS (111). Pigs with inducible tumors have previously been imaged with CT and MRI, which is proof of concept that these imaging techniques can be performed on pigs (110).

There are also practices that involve targeted imaging of tumors using small peptides and molecules. SCID mice have previously been used for such studies for ovarian (112), nasopharyngeal, breast (113), hepatic (114), lung cancer (115), and others. SCID mice are useful animal models for proof of concept studies that certain molecules and peptides can specifically bind to certain tumor types. After preliminary testing has been completed in mice, SCID pig models engrafted with human cells could then be used for testing these targeting techniques with respective imaging equipment that would be used in the clinics. As an example, human ovarian carcinomas expressing high levels of Claudin 3/4 expression will grow in SCID pigs (45). A *Clostridium perfringens enterotoxin* (CPE)

peptide can specifically bind to Claudin 3/4 (112, 116), and such a SCID pig ovarian cancer model can be used as an imaging and therapeutic target of the CPE peptide in targeting ovarian carcinomas in such a way that it is translatable to human patients.

Development of Patient Derived Xenograft Models in SCID Pigs for Personalized Drug Testing

Since SCID pigs have previously been shown to accept xenografts of human cancer cells (13), as well as pluripotent stem cells (24, 46), it would be expected that they would also accept solid tumor tissues as well. Patient derived xenograft (PDX) and cell derived xenograft models have previously been utilized in SCID mouse models for patient specific drug testing (117). SCID pig models can also be developed for these purposes. Due to higher similarity in metabolism between humans and pig (32) compared to mice, drug responses in the pig would likely lead to more directly comparable responses to those that would be found in humans (33). Additionally, the size of the pig would also allow representative drug doses to be tested that could be applied to future doses for human patients.

CONCLUDING REMARKS

Here we have described many of the novel uses of SCID pigs in oncology research involving the use of xenotransplantation of human tumor tissues, HSCs, and lymphoid tissues. The full potential of these animals will be realized when biocontainment facilities are more readily available and survivability of SCID pigs improved. Additionally, dissemination of handling protocols will be essential to prolonging the lives of these animals for long-term studies.

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Research groups generating SCID pigs are at the forefront of creating a new animal model that can be used for translational preclinical research. We have learned an incredible amount of information by use of small animal mouse models for cancer research. However, in order for therapies to be developed and tested thoroughly, they now need to be evaluated in a larger animal model that better represents human disease states and which can provide realistic opportunities for improved modeling of imaging and surgical approaches. As such, we believe that SCID pig models will provide a foundation for researchers to gain valuable and translational results to improve patient outcomes in a clinical setting.

AUTHOR CONTRIBUTIONS

AB wrote manuscript and designed figures. JC and CL edited and revised manuscript. CT wrote, edited, and revised manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2018.00559/full#supplementary-material>

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